The Effects of Estrogen on the Growth and Tuberization of Potato Plants (*Solanum tuberosum* cv. 'Iwa') Grown in Liquid Tissue Culture Media

A thesis

submitted in partial fulfilment

of the requirements for the Degree

of

Master of Science in Plant Biotechnology

at the

University of Canterbury

by

Greta Brown

University of Canterbury

School of Biological Sciences

2006

Table of Contents

Page Number

List of Figures: Page

List of Tables:

List of Plates:

List of Diagrams:

ABSTRACT

Mammalian estrogens and estrogen-like compounds known as xeno-estrogens are being found in and excreted into the environment in ever increasing amounts. The xeno-estrogen DDE has been found at high concentrations of 1-5 mg/kg of soil (Aislabie et. al, 1997). These estrogens and xeno-estrogens are having a devastating effect on animal-life, yet little is known or understood on the effects of estrogens on plant-life. Thus it is important to determine what effects (if any) estrogens may have on plants. Other research has shown that estrogen has an effect on plants grown *in vitro* (Janeczko and Skoczowski, 2005). This research aims to help increase the amount of information on what effects estrogens may have on plants. In this study, the effects of mammalian estrogens (17-β-estradiol, estrone and estriol) on the growth and tuberization of potato plants (*Solanum tuberosum* L. cv 'Iwa') grown in liquid tissue culture medium are presented. It was found that at even 0.1 mg/L of estrogen, root growth of the plants was diminished and at 10 mg/L of estrogen, plant deformity was apparent and callus growth induced. Acid phosphatase activity of the plants was increased with the addition of 0.1 mg/L and 1 mg/L of estrogen but then decreased with the addition of 10 mg/L of estrogen. Tuber production was slightly reduced in plants treated with estrogen compared to the control.

1. Introduction

1.1 Estrogen

1.1.1 What Estrogen Is

Estrogen is the common name given to a group of female steroid sex hormones produced by humans and other mammals (also birds). The primary role of estrogen in mammals is to help develop and maintain sex characteristics in females (Eckert Animal Physiology, 2001).

The biosynthetic source of estrogen is cholesterol (Biochemistry, 1990). Estrogens are released into the body via the endocrine system. The endocrine system is a group of organs and tissues in mammals (and birds) bodies which collectively release hormones into the body and blood stream (Funk and Wagnall's New Encyclopaedia, 1990). The endocrine glands are ductless and secrete estrogen directly into the blood stream of the animal. A feature of estrogen is that it is not stored after synthesis, so as soon as it is synthesised it is released into the blood stream (Biochemistry, 1990). The level of estrogen circulating in the body is controlled by the rate of production and this rate of production along with the release of estrogen into the body is controlled by the brain. Neuro-hormones released by the brain (from the hypothalamus and pituitary) target glands in the endocrine system to synthesise and release estrogen (Eckert Animal

Physiology, 2001). Estrogens in the blood stream enter target cells of the body through macromolecular cell receptors. So estrogen binds to the cell receptors which are located in the plasma membrane or occasionally the interior of the target cell (Biochemistry, 1990).

There are three main types of mammalian estrogen. They are β-estradiol (also called estradiol and 17- β-estradiol), estrone and estriol. Estradiol is formed by the ovaries, testis, placenta and possibly the adrenal cortex. Estrone and estriol are also formed by the placenta and other endocrine organs of the body (Merck Index 11th Ed. 1989). All of these three estrogens have a basic sterane carbon skeleton (Janeczko and Skoczowski, 2005).

1.1.2 The Chemical Structure and Attributes of Estrogen

1.1.2.1 β-Estradiol

Other names for β-estradiol are 17- β-estradiol and estradiol. The chemical formula for β-estradiol is $C_{18}H_{24}O_2$. See diagram one for the chemical structure of β-estradiol.

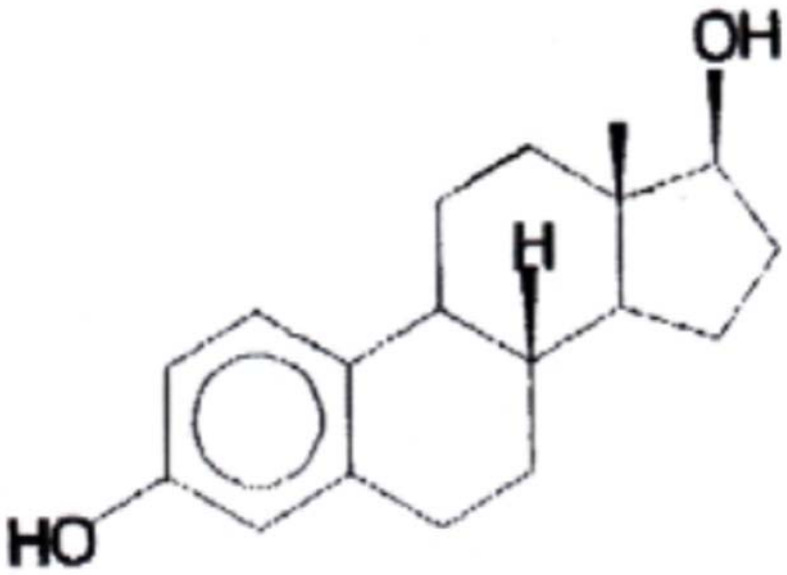


Diagram 1. The chemical structure of β-estradiol.

The molecular weight of β-estradiol is 272.37 and it is 79.37 % carbon, 8.88 % hydrogen and 11.75 % oxygen (Merck Index 11th Ed. 1989). β-estradiol is the most potent mammalian hormone (Shaw, 2001; Merck Index 11th Ed. 1989). It is stable in air, has a melting point of 173-179˚C and is almost insoluble in water. It is freely soluble in 100 % (v/v) ethanol, other alcohols and organic solvents (Merck Index 11th Ed. 1989). β-estradiol has been successfully isolated from many sources such as urine from pregnant mares and follicular liquor of sow ovaries.

1.1.2.2 Estrone

Estrone is a metabolite of β-estradiol but is not as potent i.e. it is less biologically active than β-estradiol. Estrone's chemical formula is $C_{18}H_{22}O_2$ (see diagram two) and is 79.96 % carbon, 8.20 % hydrogen and 11.84 % oxygen and has a molecular weight of 270.36 (Merck Index 11th Ed. 1989).

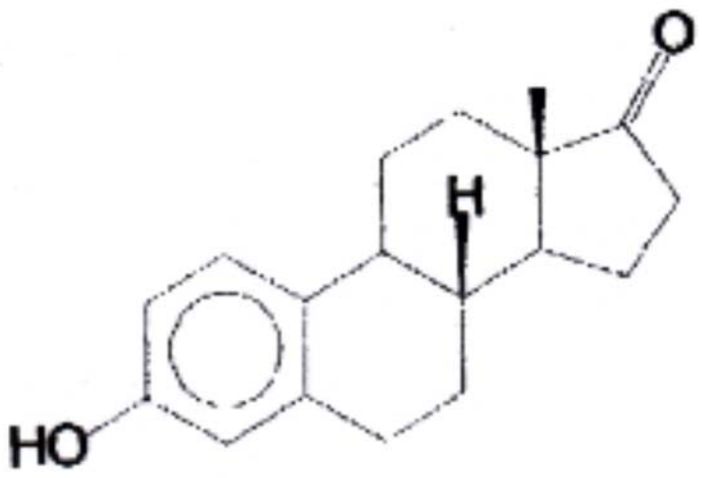


Diagram 2. The chemical structure of estrone.

Estrone is soluble in alcohol (i.e. 100 % ethanol v/v). Estrone can be isolated from urine of pregnant women, pregnant mares, bulls and stallions. It can also be isolated from follicular liquor of a large range of animals and can be found in oil from palm kernels (Merck Index 11th Ed. 1989; Shore and Shemesh, 2003).

1.1.2.3 Estriol

Estriol, like estrone is a metabolite of β-estradiol and is a much less potent hormone than β-estradiol. Its chemical formula is $C_{18}H_{24}O_3$. See diagram three.

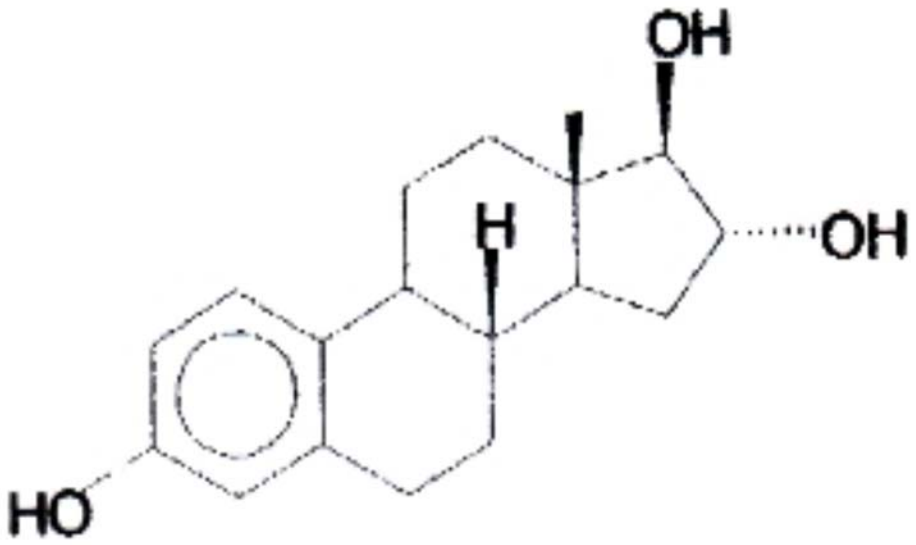


Diagram 3. The chemical structure of estriol.

Estriol has a molecular weight of 288.37 and is 74.97 % carbon, 8.39 % hydrogen and 16.64 % oxygen. Estriol is nearly insoluble in water but is soluble in alcohol, vegetable oils and ether. During pregnancy large amounts of estriol are produced by the placenta

(Merck Index 11th Ed. 1989). Estriol can be isolated from human urine (Shore and Shemesh, 2003) and plant sources (Merck Index 11th Ed. 1989).

1.1.3 Estrogen and the Environment

1.1.3.1 Location and Sources

Mammalian estrogens (estradiol, estrone and estriol) are commonly occurring substances in the environment. Estrogen has been detected in the ocean, lakes, rivers and streams (Atkinson et. al. 2003). They are easily found in water-run-off, ground water, soil and have been isolated in plants (Shore and Shemesh, 2003). Estrogen has been isolated in plant seeds, flowers, pollen, roots, shoots, oil, kernels and bulbs. Mammalian estrogen can be isolated throughout entire plants (Janeczko and Skoczowski, 2005).

Common contributors of mammalian estrogens to the environment are humans, cows, horses, poultry and other large mammals (Shore et. al., 1998; Shore and Shemesh, 2003). Sewage from humans and effluent from mammals and poultry are how most of the estrogens enter into the environment (Shore and Shemesh, 2003; Atkinson et. al, 2003). Sewage can be pumped into the oceans and animal effluent can absorb straight into the soil and waterways. Table one shows some values for how much estrogen is excreted (in either urine or faeces) by some mammals per 24 hrs or per kg of excrement. Pregnant women can excrete up to 1000 times more estrogen in a 24 hr time period than non-pregnant females (Shore and Shemesh, 2003).

Mammal	B-estradiol	Estrone	Estriol
Human Male	1.5 µg/ 24hr	3 µg/ 24hr	
Human Female	0.3-5 µg/ 24hr	2-20 µg/ 24hr	
Pregnant Female	26 mg/ 24hr	37 mg/ 24hr	3-65 µg/ 24hr
Milk cows (slurry)	170-1230 µg/ kg	255-640 µg/ kg	
Milk cows (pregnant, urine)	41mg/ 24hr or 1.3 mg/kg	44mg/ 24hr or 1.4 mg/kg	
Bulls (slurry)	<2 µg/ kg	< 2µg/ kg	
Pigs (slurry)	2-64 µg/ kg	2-84 µg/ kg	
Horses (pregnant, urine).		200-800 mg/24hr or 400 mg/kg *	

Table 1. Some values on the amount of estrogen excreted by some mammals (values are from Shore and Shemesh, 2003). Note the massive increase in estrogen between non-pregnant and pregnant females. *This value represents estrone + equilin. The human hormone replacement drug known as Premarin comes from this source.

In one year the average person produces about 50 litres of faeces and 500 litres of urine (www.ecosanres.org), so that works outs at ~0.137 L of faeces and ~1.37 L of urine in one day, which is a total of ~1.507 litres of excrement a day. So a pregnant woman would be excreting up to ~17.25 mg/L of β-estradiol and ~24.55 mg/L a day of estrone.

Up to 50 % of estrogen present in human sewage can be from pharmaceuticals (i.e. hormone replacement pills) and faecal estrogens (Shore and Shemesh, 2003).

1.1.3.2 Concentrations of Estrogen in the Environment

Estrogen has been found at concentrations of 40 pg/L (almost undetectable) up to ~2000 pg/L – 15 ng/L in the ocean and higher concentrations of estrogen have been found closer to sewage outlets (Atkinson et. al 2003; Fenske et. al, 2005). Sewage that has been able to enter the environment was found to have estrogen at the concentration of 22,000 $\pm$ 2200 pg/L and some sewage contained up to 77,000 $\pm$ 14,000 pg/L of estrone (Atkinson et. al, 2003) and 200 ng/L (0.2 micro-grams) of combined estrogens (Matsuda et. al, 2001). Estradiol has been found at concentrations of 150 ng/kg of soil in a field with no manure and at 650 ng/kg of soil in areas containing manure (Shore and Shemesh, 2003). Estrogen in run-off water after rain, was found at concentrations of 1-3 μg/L and at 25 ng/L in pond water that received this run-off. Also the estrogen in the pond water had a half-life of ~2.5 months; this means that the estrogen is taking some time to degrade (Shore and Shemesh, 2003). Low levels of estrogen (<0.5-5 ng/L) have been found in wells and springs; higher levels of estrogen (6-166 ng/L) have been detected in wetlands and

aquifers (Shore and Shemesh, 2003). The half life of estrogen (depending on its location) varies between 0.37 days and ~2.5 months (possibly longer), so estrogen can be degraded very slowly (Shore and Shemesh, 2003).

There are a few scientific methods to estimate the concentrations of mammalian estrogens in plants i.e. the Krober colour reaction and radio-immunoassays (Janeczko and Skoczowski, 2005). Both of these methods can detect estrogen to minute quantities such as 1 part per billion. β-estradiol was detected at 8-35 pg/g (a very low concentration) in the pollen and style of *Zea mays* L. and *Brassica campestris* L. (Janeczko and Skoczowski, 2005). It is important to note that the concentrations of estrogen can vary greatly depending on the developmental stages of the plants, different organs in the plants and also amongst different species and cultivars. Some plants may not even assimilate and maintain mammalian estrogen (Janeczko and Skoczowski, 2005). Estrogen has been isolated at concentrations of 2.5 - 4.5 µg/kg in *Punica granatum* L. plants and up to 50 µg/100 g (dry weight) in *Salvia splendens* plants. Estrone was found to be at concentrations of 5.13-5.25 mg /kg in *Hyphaene thebaica* L. plant kernels and 8.1 mg/ 240 g in the kernels of *Olea europea* L. Estrone was also present in *Zea mays* L. oil at 4 µg/100 ml and β-estradiol was extracted at 2-10 µg/kg (fresh weight) in the seeds and leaves of *Phaseolus vulgaris* L. (Janeczko and Skoczowski, 2005).

1.1.3.3 Some Effects of Estrogen on the Environment

Estrogen has been found to affect wildlife in the environment. A study carried out to see the effects of estrogen on Zebra fish (*Danio rerio*) found that estrogen arrested the development of the male Zebra fishes' gonads (Fenske et. al, 2005). It is also thought that too much estrogen may have a feminising effect on human males and reduce their reproductive capabilities (Shaw, 2001).

1.1.3.4 Research Findings on the Effects of Estrogen on Plants Growth

Since mammalian estrogens have been found in the environment and isolated in plants, a very valid question is posed: what effects are estrogens having on plants and their functions? It has been found that mammalian estrogen is active in some plants i.e. female mulberries (Janeczko and Skoczowski, 2005). So if estrogenic activity is occurring in plants then mammalian estrogen maybe affecting them.

In a study carried out on the effects of estrone and β-estradiol on the vegetative growth of alfalfa (*Medicago sativa*), it was found that with very low concentrations of these two estrogens (0.005-0.5 μg/L), the dry weight of both the roots and shoots increased and with much higher concentrations of estrogen (50-500 μg/L) the alfalfa growth decreased (Shore et. al, 1992). It has also been found that estrogen can decrease early root growth in mung beans (*Phaseolus aureus*) (Guan and Roddick, 1988). It has been shown that even

though sunflower seedlings shoot growth increased when treated with β-estradiol, root growth was inhibited (Janeczko and Skoczowski, 2005).

An important finding which is very relevant to the results presented in this thesis is the effects of estrogen on tomato plants. Both tomato plants and potato plants belong to the nightshade family and therefore it is possible that the effects of estrogen on the growth of tomato plants and potato plants will be similar. It was found that estrone and β-estradiol reduced the growth and number of roots of tomato seedlings (Janeczko and Skoczowski, 2005).

Mammalian estrogen does not only effect root and shoot growth in plants it can also cause callus growth, effect the morphology of plants, induce flowering, alter sex expression in plants and stimulate pollen tube growth. It was reported that callus growth in tissue cultured *Daucus carota* L. increased by up to 100 % when these plants were treated with β-estradiol (Janeczko and Skoczowski, 2005). Tomato plants treated with mammalian estrogens exhibited morphological abnormalities including leaf rolling and downward curling in the leaves (epinasty) (Janeczko and Skoczowski, 2005). In 2003 Anna Janeczko and colleagues discovered that estrogen stimulated flowering in *Arabidopsis thaliana*, but they also found that β-estradiol, estrone and estriol delayed the arrival of the first flower bud. They also established that estrogen inhibited generative development in *A. thaliana* (Janeczko et. al, 2003). Mammalian estrogen is reported to have increased the amount of female flowers compared to male flowers in cucumbers and *Ecballium elaterium* L. (Janeczko and Skoczowski, 2005). It has been shown that

estrogen stimulated pollen germination and pollen tube elongation in tobacco plants grown *in vitro* (Ylstra et. al, 1995).

To summarize, mammalian estrogen affects the growth of plants. It especially affects the roots of plants, often decreasing the amount and size of the roots. Estrogen can stimulate the abnormal growth of plant tissue (callus) and alter the morphology of plants.

1.1.4 Estrogen-like Compounds

There are many estrogen-like compounds in the environment and they are known as xeno-estrogens. Xeno-estrogens mimic the most potent estrogen which is 17-β-estradiol (Shaw, 2001). Xeno-estrogens enter the cells of humans, animals and plants via the 17-β-estradiol cell receptor (Thomson, 2005). Receptors for 17-β-estradiol have been located in plants eg. *Gladiolus primulinus* Bak. (Janeczko and Skoczowski, 2005). Xeno-estrogens are in compounds such as pesticides, herbicides and plastics. A xeno-estrogen very relevant to the Canterbury region in New Zealand is dichlorodiphenyltrichloroethane also called DDT (Aislabie et. al, 1997; Thomson et. al, 2003). Xeno-estrogens can range in concentrations and can take years to degrade. For example in soils in Canterbury (NZ) it is common to find 1-5 mg/kg of dichlorodiphenyldichloroethylene also called DDE (a metabolite of DDT) and DDT has not been used for years in this soil (Aislabie et. al 1997).

Xeno-estrogens are having a devastating effect on wild life in the environment. There is limited data (if any) on the effects of xeno-estrogens on plants but there are large amounts of evidence to suggest that xeno-estrogens are affecting fauna and possibly humans. It was discovered in the early 1990's that alligators in Lake Apopka in Florida USA had shorter penises that alligators in a nearby lake called Lake Woodruff. Lake Apopka was fed by rivers downstream of agricultural and industrial areas and was thus quite polluted compared to Lake Woodruff. It was found that Lake Apopka contained DDE (a metabolite of DDT) and that DDE was stored in the alligators' body fat. Thus the short penises of the alligators were attributed to the metabolite of the xeno-estrogen DDT (Thomson et. al, 2003). It is thought that xeno-estrogens present in food may reduce the sperm count in human males (Shaw, 2001). It has been found that female marine gastropods exposed to tributyltin oxide (TBTO) were masculinized, fish exposed to xeno-estrogens from industrial sources have reproductive abnormalities and male roaches were feminised (Thomson, 2005). Amphibians, reptiles and birds also have developed abnormal reproductive systems and mammals have showed signs of abnormal reproduction and lowered immune function (Thomson, 2005). Estrogen-like compounds called xeno-estrogens are affecting the environment and these xeno-estrogens function the same as 17-β-estradiol.

1.2 Potatoes

1.2.1 History of the Potato

Potatoes that produce edible starchy tubers are from the family Solanaceae (the nightshade family including tomatoes). They are from the genus *Solanum* and the species used in this research is *Solanum tuberosum* (Encyclopaedia Britannica online version). *Solanum tuberosum* plants produce a white tuber. There are approximately 150 species of potatoes belonging to the *Solanum* genus. It is thought that the potato originated in the Peruvian Andes of South America and the Spanish army discovered them in their conquest of Peru in 1524 (Potato Association of America Handbook, 2005). In the late 16th century the Spanish bought potatoes back to Spain and in about the 18th century potatoes were introduced to North America and Ireland. The introduction of potatoes as food in the USA was accepted very slowly but by 1840 they became commonplace and now they are eaten worldwide. Throughout the world these days billions of potatoes are produced and consumed yearly (Potato Association of America Handbook, 2005). The potato industry is now a multi-billion dollar industry.

1.2.2 Morphology and Characteristics of Potatoes

1.2.2.1 The Plant

The potato plant can grow to be a large bushy plant up to ~1 meter high. Potato plants have medium sized (up to ~8 cm long) green leaves, smallish white flowers and tubers than grow underground (see diagram four on the next page). Potato plants are annuals and are dicotyledons. Even though they are annuals their tubers can sprout and grow new plants. The potato plant stems have their vascular bundles in a circular pattern and have layers of xylem and phloem (Potato Association of America Handbook, 2005).

Potato plants can be susceptible to a fungus called *Phytophthora infestans* or more commonly known as late potato blight, they are also susceptible to another fungus called *Alternaria solani* or the early blight. These blights can cause the stems, leaves and tubers to rot. Potatoes also suffer attacks from insect pests such as the Colorado Beetle (*Leptinotarsa decemlineatum)* (Funk and Wagnall's New Encyclopaedia, 1990).

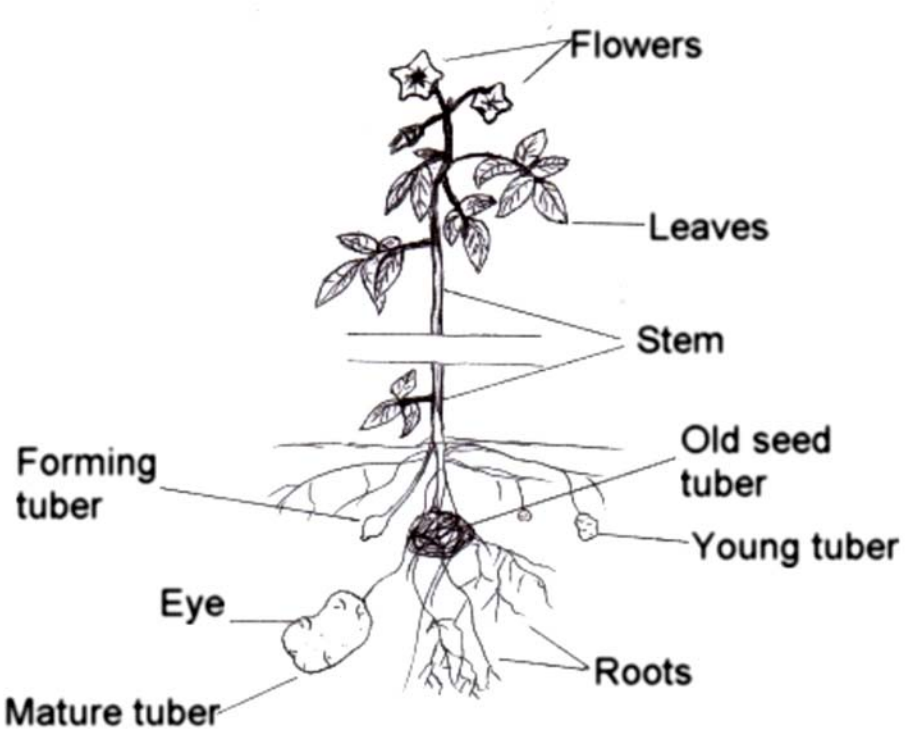


Diagram 4. The general features of a fully grown potato plant (Adapted from the Potato Association of America Handbook, 2005).

1.2.2.2 The Tuber

The potato tuber (see diagram five, next page) is the edible part of the potato plant. The tuber is an enlarged section of a stem known as the rhizome (or stolon) which is formed

underground (Potato Association of America Handbook, 2005). A freshly dug tuber contains ~78 % water, ~18 % starch, ~2.2 % protein, ~1 % ash and ~0.1 % fat; roughly 75 % of the dry weight of a potato tuber is carbohydrate. The starch from tubers can be used in alcohol and adhesives (Funk and Wagnall's New Encyclopaedia, 1990).

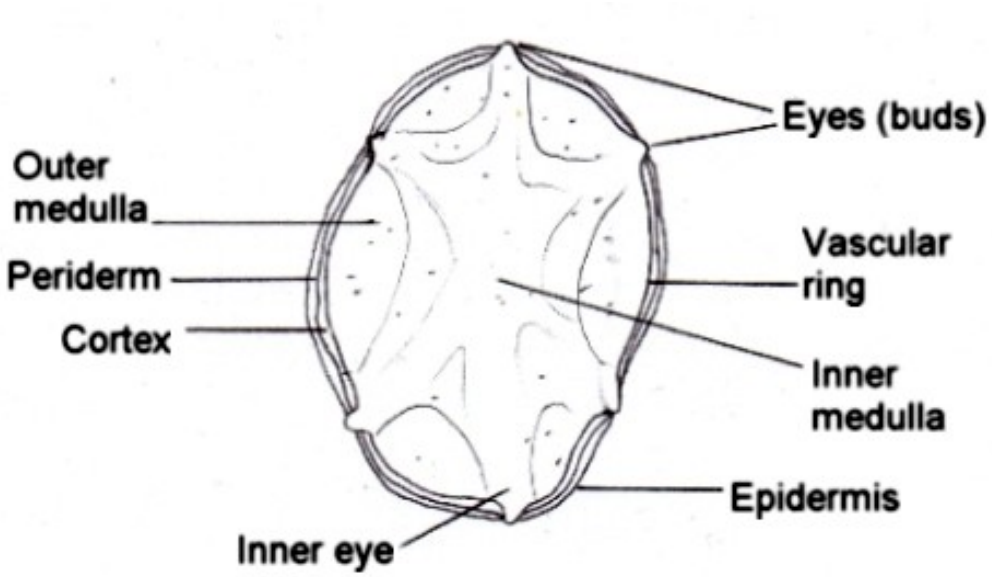


Diagram 5. The structure of a potato tuber, note how the inner medulla branches to the eye (Adapted from the Potato Association of America Handbook, 2005).

Since the tubers are formed out of stems, they retain the features of normal stems, but on the tubers the buds are called eyes. The eyes on a tuber are placed in a spiral pattern and are mainly located toward the apical or seed end of the tuber (though the eyes can be found on other areas of a tuber). The eyes are where new shoots are sprouted and grow from, and thus a whole new potato plant grows which will produce a new round of tubers (Potato Association of America Handbook, 2005). The colour (pigmented) section of a

tuber is located in the periderm which is just below the epidermis. A small number of potato breeds produce tubers with coloured pigmentation throughout the entire tuber (Potato Association of America Handbook, 2005).

1.2.3 Potatoes as a Scientific Plant Model

Potato plants have been used as a plant model in many scientific experiments. For example researchers used *Solanum tuberosum* to investigate the effects of sucrose on polyploidization in early callus cultures (Pijnacker and Ferwerda, 1990). Potato plants have also been used to investigate sucrose utilization (Yu et. al, 2000) and to find the effects of ethylene on the tuberization of potato cuttings (Vreugdenhil and Van Dijk, 1989). So this research is using potato (*Solanum tuberosum*) as a plant model because they are easy to grow and work with, and have been successfully used in many previous research experiments.

1.3 Plant Tissue Culture

1.3.1 The Basis of Plant Tissue Culture

Plant tissue culture, also known as micro-propagation or clonal propagation, is the technique of taking a piece of plant tissue (called an explant) or plant cells and placing them in/on specialized growth medium to grow an entire new plant, plant pieces, cells or callus (which ever is desired) from that one piece of plant tissue or plant cells. The new plant will have an identical genetic make-up to the plant from which the explant/cells

were sourced (Biology of Plants 6th Ed, 1999). Plant tissue culture relies on a characteristic in plant cells (excluding cells that lose their nuclei i.e. sieve tube phloem cells) known as totipotency. Totipotency is the ability of a plant cell to retain all of the genetic material that is needed to develop an entire plant (Plant Physiology 2nd Ed, 1998). See plate one for an example of entire plants grown from explants.

Plate 1. These entire potato plants grew from small sections of potato stems. These plants are ~ 8 cm high.

Tissue culturing of plants needs to be done in a sterile environment because fungal and microbial infections can kill the plants (tissue culture medium is a perfect environment for fungal and bacterial growth). With tissue culture is it possible to control the environmental conditions in which the plant is growing (Biology of Plants 6th Ed, 1999). This is an advantage because it is usually impossible to control what is in soil but you can control everything that is present in tissue culture medium.

The ingredients of tissue culture medium can alter how the explant/cells will grow. Some media will cause the explant/ cells to be grown into callus, others will cause only root or shoot growth and other media will cause an entire, healthy plant to grow (Plant Physiology 2nd Ed, 1998). Also temperature and lighting conditions will alter growth. See plate two for how growth conditions can alter the resulting plant.

Plate 2. These potato plants originally looked like those in plate one, but after the sucrose concentration in their medium was altered and they were placed in the dark they formed tubers and lost that very green leaf colour. The plants in plate one could not form tubers as they were not exposed to the correct environment for tuberization.

T. Murashige and F. Skoog developed a very efficient tissue culture medium that is now used widely throughout experiments working with plant tissue culture. They found that altering the ingredients in the tissue culture media altered the growth of tobacco tissue

cultures. A simple example of this is that the dry weight of tobacco plants was altered with the amount of sucrose added. They found that plants grown with 2 % sucrose in the tissue culture media had an average dry weight of 0.44 g/flask, 3 % sucrose produced plants with a dry weight of 0.67 g/flask and 4 % sucrose in the tissue culture medium produced plants with a dry weight of 0.62 g/flask (Murashige and Skoog, 1962). This shows that not enough or too much of an ingredient can alter the growth of tissue cultured plants. The media designed by T. Murashige and F. Skoog is so successful because it contains major salts, minor salts, organic supplements, iron supplements and carbohydrates (sucrose). Adding plant hormones/ growth regulators such as gibberellin, IAA (indoleacetic acid) and kinetin could exert great influence on the growth of cultured plants (Murashige and Skoog, 1962). The pH of a tissue culture medium is often adjusted to 5.6-5.8 as it is also thought to be able to influence the growth of the plant materials in culture (Dixon, 1985).

1.3.2 Major Types of Plant Tissue Culture

The major types of plant tissue culture are organ/embryo culture, cell suspension culture, culture of plant tissue, callus culture and protoplast culture (Biology of Plants 6th Ed, 1999). Organ/embryo culture is where an organ eg. node, or plant embryo (i.e. from a seed) is taken and grown in tissue culture medium. Cell suspension culture is where pieces of plant tissue are broken into cells and these cells grow and divide; these cells can be placed in growth medium and entire plants will grow (Biology of Plants 6th Ed, 1999). Culture of plant tissue is where a piece of plant tissue i.e. a section of stem is taken and

placed in specialized growth medium and a new plant develops. Callus culture is where a plant is injured and a callus forms, then cells from this callus are taken and grown in tissue culture medium. The callus cells can be induced to grow entire plants, roots or shoots, or new callus clumps (Biology of Plants 6th Ed, 1999). A very important tissue culture method is protoplast culturing. Protoplasts are plant cells which have had their cell walls removed. With the cell wall gone two protoplasts from two different plants can be fused together to create a somatic hybrid cell (Biology of Plants 6th Ed, 1999). The somatic hybrid cells can then be treated and they will re-grow cell walls and a new entire plant will grow. This new plant will have attributes from both of the original plants (Biology of Plants 6th Ed, 1999). This is useful because protoplast culture can be used to create novel plants that display desired attributes from two different plants which are not normally sexually compatible. An example of a protoplast culture is "*Arabidobrassica*" which is a plant resulting from the protoplast culture of cells from *Arabidopsis thaliana* and *Brassica campestris* (Gleba and Hoffmann, 1980).

1.3.3 Uses of Plant Tissue Culture

Tissue culture is very useful to build up stocks of non-contaminated plants for later use (Bieleski, 2000). Tissue culture also provides a viable way to produce large amounts of genetically identical plants (Biology of Plants 6th Ed, 1999). This is important for industries that require large amounts of very similar plants i.e. in Canada they use tissue cultured plants for forestry (www.nrcan.gc.ca), and it is also very important for conservation purposes. If a plant is nearly extinct it may be possible to culture it and save

it from extinction and in 1991 S. Seeni and P. Latha were able to regenerate foliage of *Renanthera imschootiana* Rolfe (Red Vanda from the orchid family) which is an endangered plant (Seeni and Latha, 1991). Tissue culture is also an easy way to produce novel plants with desired attributes i.e. plant crops that are pest and drought resistant (Bieleski, 2000).

Importantly, tissue culture is a very useful technique to examine effects of certain entities on plants. Tissue culture is useful for this purpose because you can account for all factors in the plant's environment, where as when using plants grown in soil you cannot be sure of exactly what the soil contains. So researchers have found tissue culture useful for many studies. An example of this is finding the effects of salt and drought stress on the activity of acid phosphatase in alfalfa plants. Since the alfalfa plants were grown in tissue culture medium the amount of salt (NaCl) and water could be controlled and the concentration of salt in the media was always a known quantity (Ehsanpour and Amini, 2003). With soil there may be some extra salt present which is not accounted for. Also when it came time to test how much acid phosphatase had been released by the alfalfa into the medium, the researchers could be sure that any acid phosphatase present had to have come from the plant because they had not added any. If the plants had been grown in soil some of the acid phosphatase may have been released by microbes living in the soil. So using tissue culture for experiments on plants is very useful because it eliminates the problem of dealing with unknown factors that would be an issue if soil was the growth medium.

1.4 Acid Phosphatase

1.4.1 What is Acid Phosphatase?

The name acid phosphatase is given to a group of enzymes which catalyse the hydrolysis of phosphate esters (Duff et. al, 1994). The full name of acid phosphatase (also known as APase (Turner and Plaxton, 2001)), is orthophosphoric-monoester phosphohydrolase (Turner and Plaxton, 2001) (Duff et. al, 1989).

The pH range for acid phosphatase (APase) is below pH 7.0 (Duff et. al, 1994) and its optimum activity range is between about pH 5.0 and pH 6.0 (Duff et. al, 1989). There are also alkaline phosphatases, whose optimal pH for catalysing is above pH 7.0 (Duff et. al, 1994). Since the experiment in this thesis is using only the acid phosphatase, the alkaline phosphatases will not be the focus here.

There are two distinct types of plant APases: the first are non-specific enzymes, this is because they appear to have no substrate specificity. The second type of plant APases are specialised because they seem to have fairly specific substrates (Duff et. al, 1994). An example of a specialised APase enzyme is the phosphoenolpyruvate phosphatase in *Brassica nigra* which can "bypass the adenosine diphosphate dependent pyruvate kinase reaction" during long-time periods of phosphate starvation (Duff et. al, 1989).

The above two types of APases can then be split further into three classes. First are the APases of low molecular weights (18-20 kDa). The second class is the high molecular weight group with MW of 45-60 kDa per sub-unit of APase. The third class are called purple acid phosphatases and have a binuclear iron-zinc centre (http://gamma.mbb.ki.se) (Duff et. al, 1994).

1.4.2 The Activation and Inhibition of Acid Phosphatase

Environmental stresses are reported to cause an increase in APase activity (i.e. they activate/induce APases). These stresses include water deficiency, lack of phosphate (P_i) and excessive amounts of salt (Duff et. al, 1989; Staswick et. al, 1994; Ehsanpour and Amini, 2003). APase can also be activated by divalent cations (eg. Mg^{2+}, Ni^{2+} and Ca^{2+}) (Duff et. al, 1994), but these cations, along with large amounts of P_i can be inhibitory (Angosto et. al 1988). So if there are large amounts of P_i it will reduce the amount of APase being activated and produced.

1.4.3 The Role of Acid Phosphatase

In a plant's natural environment there are many growth limiting factors, but a major one is the availability of phosphate (P_i) (Baldwin et. al, 2001). P_i (phosphate) is important for metabolic regulation and transfer of energy; it is "also and important structural constituent of many biomolecules" (Duff et. al, 1994; Ehsanpour and Amini, 2003). The type of phosphate which plants prefer to absorb is the inorganic orthophosphate anion

(Baldwin et. al, 2001; Duff et. al, 1994). Even though plants prefer inorganic phosphate, a high percentage (up to 80%) of this nutrient is to be found in an organic form (Baldwin et. al, 2001). Because a high amount of the required P_i is in an organic form, plants need a way to turn organic phosphate into inorganic phosphate so that they can assimilate this required nutrient. For this role plants produce acid phosphatases (APases) (Baldwin et. al, 2001). The plants secrete APase into their growing medium (which is usually soil) to catalyse the hydrolysis of organic phosphate and other phosphorylated substrates into the usable inorganic phosphate (Xiaolong et. al, 2001; Baldwin et. al, 2001; Duff et. al, 1989). In summary, plants produce APase to break down unusable phosphates into the usable inorganic phosphate.

1.4.4 The Sources of Acid Phosphatase(s)

Acid phosphatase (APase) is a ubiquitous enzyme (Duff et. al, 1989), which means that it can be found in a wide range of species and tissues (Hiroshi et. al, 2001). APases are found in micro-organisms (eg. bacteria, yeast and algae (Bieleski, 1973)), plants and animals (Hiroshi et. al, 2001; Bieleski, 1973). APase in animal cells is found in the organelle called the lysosome (Bieleski, 1973). Since this research is using plants, the focus here will be on where APase is found in plants and its sources. APases are found in most plants if not all eg. potatoes, tomatoes, lupins (Baldwin et. al, 2001), brassicas (Duff et. al, 1989) and bananas (Turner and Plaxton, 2001). Plant APases have been found in tubers, bulbs, roots, leaves, seeds, the aleurone layer (Hiroshi et. al, 2001), suspension cells (Duff et. al, 1989) and fruit (Turner and Plaxton, 2001). Also an interesting fact is

that when the activity of APase is increased in the plant, the activity of APase also increases in the rhizosphere (Baldwin et. al, 2001). So there is APase activity around the plant roots in the micro-organisms of the rhizosphere. Plant roots exude APase into the medium that they are growing in i.e. tissue culture medium (Dakora and Philips, 2002) and soil (Tomscha et. al 2004).

1.4.5 The use of Acid Phosphatase as a Growth Indicator

In 2002 at the University of Canterbury, Naomi Ling found that the amount of acid phosphatase units in the tissue culture media increased when the growth of the potato plants increased. She found that the better the potato plants grew the more APase they released into the tissue culture medium. This is important as this research will use the amount of APase present in the tissue culture medium as an indication of the growth differences between treatments.

1.5 Protein Determination

Plants synthesize many proteins and exude many of these substances through the surface of their roots into their growth medium (Diz et. al, 2003). An example of this is the enzyme acid phosphatase which is a protein and is exuded through the plant's roots into the surrounding growth medium (Baldwin et. al, 2001). As has been previously shown in section 1.4.5, the amount of a soluble protein, specifically acid phosphatase, released by the cultured potato plant into its growth medium could be used as a growth indicator for

the plant. It is possible that the total amount of soluble proteins released by the cultured plants into their growth medium (liquid tissue culture media in this research) may also be correlated with the differences in growth of the plants in different experimental treatments. Thus it is hypothesized that the more protein in the growth medium the better the cultured plant has grown.

There are two protein determination assays outlined by Marion Bradford. The first is the standard assay which is for microgram quantities of protein above 10 μg. The second is the micro-protein assay which is for the much smaller quantities of protein (1-10 μg). These assays use a dye which binds to the protein (Bradford, 1976). When the absorbance of the sample is read, the higher the absorbance value, the greater the amount of dye that has bound to the protein in the sample. The more protein that is present in the sample, the greater the amount of dye that will be bound. The absorbance readings are then matched against absorbance readings from samples with known concentrations of protein (Bovine Serum Album is normally used) and the amount of protein in your sample can then be calculated. Many researchers use the Bradford method (Bradford, 1976) to determine the total amount of soluble protein in a sample. Mariângela Diz and colleagues used the Bradford method of protein determination to determine how much total protein was being exuded by cowpea (*Vigna unguiculata*) seeds (Diz et. al, 2003).

1.6 One-Way ANOVA

One-way ANOVA is a statistical method for analysing data. ANOVA stands for analysis of variance. One-way ANOVA will give you the mean of each treatment (from the data used), but more importantly it gives a P value, F value also called Fmax value (F=variance ratio: which is found by dividing the largest variance by the smallest variance) and the results to the Tukey HSD All-Pairwise Comparisons Test. The P value is important because if it is below 0.05 it means that the data has a trend; if it is above 0.05 it means that your data does not have a trend, i.e. there is no significant difference between each treatment in your data. If the P value is one or above in your data it means that it is not good according to one-way ANOVA.

In one-way ANOVA the F value (largest/smallest variance ratio), shows whether or not there is excessive variation in your data. If the F value from your data is below the F max value (a value given by degrees of freedom (number of replicates in the experiment minus one, i.e. n-1) and the number of treatments in the experiment), which can be found on Fmax tables, it means that your data is statistically good i.e. there is not excessive variation in your data. If the F value is above the Fmax value, then in statistical terms there is too much variance in the data for statistical comparison.

The Tukey HSD All-Pairwise Comparisons Test shows whether or not there are significant pairwise differences amongst the means of the data. If there are significant pairwise differences, it can indicate that there is a trend and where that trend is.

1.7 Aims and Objectives

The objectives of this research are to find out if mammalian estrogen will have any effects on the growth and tuberization of potato plants grown in liquid tissue culture medium, and to find out what these effects may be. Concentrations of the three chosen estrogens, namely 17-β-estradiol, estrone and estriol, will be used that cover a range of concentrations for both estrogen and xeno-estrogens that are known to exist and/or be exuded into the environment. For example a pregnant woman can exude ~24.55 mg/L of estrone in 24 hrs and 1-5 mg/kg of DDE (a metabolite of the xeno-estrogen DDT and a xeno-estrogen itself) has been extracted from Canterbury soils even years after DDT use was banned. This research also aims to use concentrations of estrogen that are in a similar range to concentrations used by other researchers i.e. 50-500 µg/L (0.05-0.5 mg/L) of estrogen (Shore et. al, 1992). The three most common estrogens have been used to see if their effect on potato plants is similar. 17-β-estradiol has been used not only to see what effects it will have but because it can also give an indication of how xeno-estrogens might affect potato plants as xeno-estrogens can mimic the action of 17-β-estradiol (Shaw, 2001). Clonal potato plants have been used because they are proven to be a valuable plant model system to probe questions concerning factors influencing plant growth and development. Since the plants are being grown in tissue culture medium instead of soil, complications from unknown factors originating from soil can be successfully avoided.

2. Materials and Methods

2.1 General

2.1.1 Plant material

The potato plants used throughout the entire project were *Solanum tuberosum* L. (cv. 'Iwa'). Stock plants were micro-propagated from existing cultures in the Plant Biotechnology Laboratory at the University of Canterbury, New Zealand. These original cultures were prepared by Dr. David Leung. All plants for the actual experimental work were taken from the stock plants.

2.1.2 Stock Plants

The stock plants were grown from nodal explants of the existing cultures in the laboratory. Three explants were placed in each tissue culture jar (the tissue culture jars were 250 ml clear polycarbonate jars), containing 40 ml of autoclaved liquid MS media (Murashige and Skoog, 1962), with 3% (w/v) sucrose. The stock plants were then grown for 4 -5 weeks (before being used) in a growth room with a 24hr photoperiod (the lighting tubes were Sylvania Gro-lux tubes), and at about 22˚C. New stock was grown every 6-8 weeks to ensure a healthy and plentiful supply of nodal materials suitable for explants.

2.1.3 Explants

The explants used for the micro-propagation (tissue culturing) of the potato plants, were nodal explants. Each explant contained two nodes. From the top of the stem, the explants were cut just below the second node. The length of the explants varied from ~10mm-~15mm. See plate three for a visual illustration.

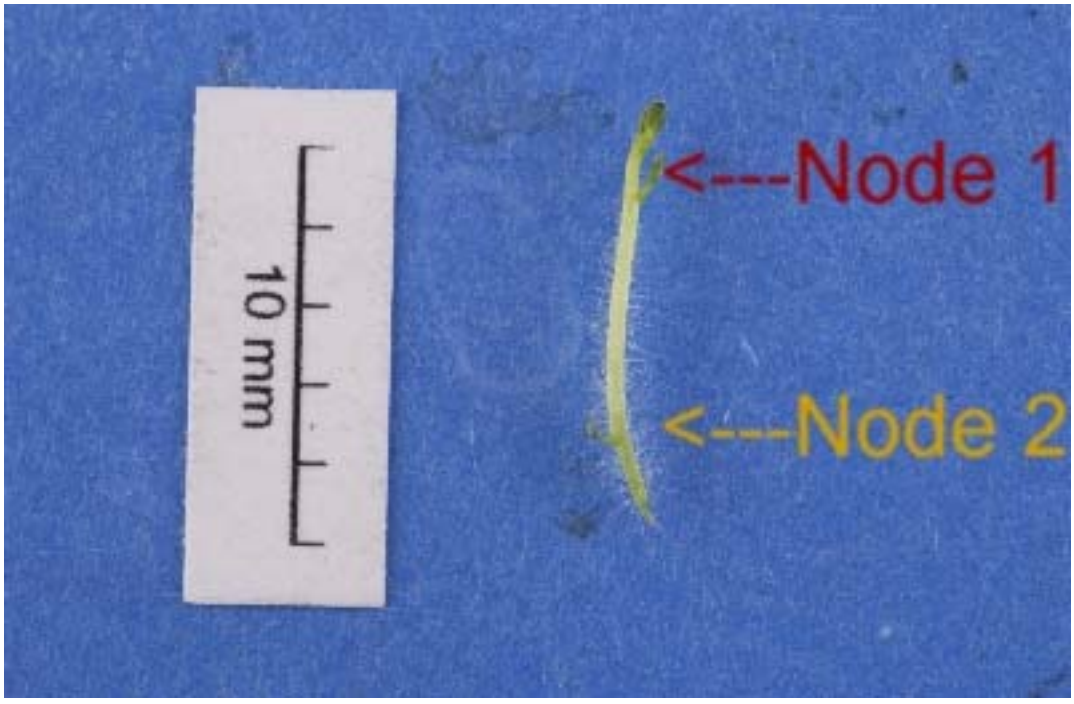


Plate 3. An example of a nodal explant. The tip of the stem is node 1 and the next leaf down is node 2.

2.1.4 Tissue Culture Media

The tissue culture medium used throughout all of the experiments is liquid MS media. The basal MS medium was designed by Toshio Murashige and Folke Skoog in 1962. (For instructions on how to make MS media see Appendix 1). The media was freshly

prepared before each experiment from stock solutions that have been stored at 4˚C in the dark. New stock solutions were made about every 3-4 months to ensure that the media were always of good quality and not too old. These stock solutions consisted of major salts, minor salts, organic nutrients and an iron solution. When the media were prepared, the correct amounts of stock solutions were combined with distilled water and the required amount of sucrose. Sucrose (Chelsea Sugar, Auckland New Zealand), was added at concentrations of either 3% (w/v) or 8% (w/v) depending on the requirements of each experiment. The pH of the solution was then adjusted to pH 5.6-5.8 using either 0.1 M NaOH (for increasing pH) or 0.1 M HCl (for decreasing pH) depending of what was required. The pH was read using a Metrohm Herisau (Switzerland) pH meter. The volume of the media was then adjusted with distilled water if necessary. The media, 40 ml per jar, were then dispensed into 250 ml polycarbonate tissue culture jars. The media were autoclaved for 20 minutes at 121˚C (the autoclaving used was wet heat sterilization). Once the media had been autoclaved, it was stored in the dark overnight. Any contaminated media were thrown out.

2.1.5 Minimizing Contamination

To avoid contamination all tissue culture work was performed inside of a laminar flow cabinet (Clyde-APAC, HWS Series). This ensured that the air in and around the work area was always sterile (i.e. all the air inside the laminar flow cabinet was sterile). The laminar flow was turned on 10-15 minutes before use so that there was time to make sure that all of the non-sterile air was blown out and that there was time to replace it with

sterilized air. Also all working surface areas inside the laminar flow cabinet were wiped down using cotton wool saturated with 70 % (v/v) ethanol before the laminar flow cabinet was used. Forceps and scalpels used for the sterile procedures were soaked in 100% (v/v) ethanol for 10 minutes before being used. All containers taken into the laminar flow cabinet were thoroughly wiped with 70 % ethanol (so were other items that were taken in, mainly pipettes). Latex gloves were worn and were also wiped with 70 % ethanol. Anything removed then put back in the laminar flow cabinet was re-wiped. All pipette tips that were required to be sterile were autoclaved.

2.2 Materials and Methods for the Effects of Estrogen on the Growth of Potato Plants Grown in Liquid Tissue Culture Media

2.2.1 Plant Material

The nodal explants required for this section of experiments were obtained from previously grown stock plants (see part 2.1.2)

2.2.2 Preparation of Estrogen Stock Solutions

Three types of estrogens, all purchased from Sigma (St. Louis, USA), were used in these experiments. They were β-estradiol (17- β-estradiol), estrone and estriol. The β-estradiol was 98% pure and both estrone and estriol were 99 % pure. Three concentrations of the estrogens were used: 0.1 mg/L, 1 mg/L and 10 mg/L. All three estrogens were in powder form which was not suitable to be added directly to liquid tissue culture media as they

would not have dissolved or dispersed evenly in the media. Therefore the stock solutions were first prepared using 100 % (v/v) ethanol (Ylstra et. al, 1995). The estrogen stock solutions were made so that the same volume of liquid would be added to each tissue culture jar even when the required concentrations of the appropriate estrogen in the media were different. For instructions on how to make up the stock solutions see Appendix 2. The stock solutions were made so that 80 µl of stock solution (of the appropriate concentration), would be added per 40 ml of liquid tissue culture media. Once the stock solutions were prepared they were placed in labelled Eppendorf tubes and stored at room temperature in a dark, dry place. New stock solutions were made as required. Sterilization of the estrogen stock solutions was not required because the ethanol used to dissolve the estrogens kept them sterile.

2.2.3 Preparation and Growing of the Potato Plants

Potato explants were grown with estrogen in 40 ml of liquid MS tissue culture media containing 3 % sucrose in each jar. There were three explants in each jar and three jars (replicates) for each treatment. There were four treatments (control, 0.1 mg/L, 1 mg/L and 10 mg/L), in an experiment. The required 80 µl of estrogen stock solution were added to the 40 ml of liquid media just before the explants were added. The control was 80 µl of 100 % ethanol (because 100 % ethanol was what had been used in the estrogen stock solutions). The estrogen solution or 100 % ethanol for the control, was carefully squirted into the liquid tissue culture media which were then swirled around to ensure

that it was evenly distributed throughout the media. This whole process was done inside a laminar flow cabinet using sterile equipment, e.g. pipette and pipette tips.

Nodal potato explants were then grown in the estrogen-containing and control media for four weeks in a growth room with a 24 hr photoperiod and a temperature of ~22˚C (the same growth room used for the stock plants). The jars containing the potato plant materials were placed randomly on the shelves in the growth room to ensure that no one particular treatment might get more light and thus affecting the results in some way. The cultures were checked weekly to see if there were any visual differences among treatments.

At the end of four weeks the experiments were terminated. The media was drained from the tissue culture jars. The media from the three jars of the same treatment were then pooled into one jar (a polycarbonate jar) and immediately placed in a -20˚C freezer to be used at a convenient time for acid phosphatase assays and protein determination assays (i.e. all the media for the control from one experiment, which is three jars worth was pooled into one jar and all the media for another concentration of estrogen would be pooled into another jar). The appearances of the potato plants were recorded and some plants photographed, thus the data can be used later for qualitative results.

The potato plants from each jar were then divided into roots and shoots (stems and leaves). The fresh weights of the roots and shoots of the three plants from a jar were then recorded. Next the roots and shoots were wrapped, separately, in tin foil and carefully

labelled to make sure that the plants from each jar and treatment were not mixed. The weight of the foil and label were recorded before the plant matter was wrapped in them. Once all the roots and shoots of the potato plants were wrapped, they were all placed in an oven (Qualtex) at ~65˚C for approximately one week. After this time they were removed and weighed. The weight of the foil and label (previously recorded) were then subtracted and the resulting number was the dry weight (see section 2.2.6, Analysis of the Raw Data, for how this data was used). This experiment was carried out twice using β-estradiol as the estrogen, three times using estrone and three times using estriol as the estrogen. The first time using estrone and estriol as the estrogens were trial runs to test that the concentrations of estrogen chosen would be suitable for this experiment. Since they were, the data from these trial runs has also been included in the results.

2.2.4 Acid Phosphatase (APase) Assays

APase assays were performed on all of the media collected from the growth experiments. The required media for the assays (for example: all treatments from the first β-estradiol growth experiment), were removed from the -20˚C freezer and placed on ice (which was held in a polystyrene ice box) to melt.

Once the media had melted, it was very gently shaken to make sure the enzyme was evenly dispersed throughout. Six Eppendorf tubes were required for each treatment, so there were 24 tubes for each batch of media. For each treatment, there were three replicate enzyme reaction mixtures and three enzyme reaction control mixtures. The

following is the procedure of a typical round of enzyme assay for one treatment (eg. 10 mg/L for the 1st β-estradiol experiment). 0.5 ml of citrate buffer pH 5.0 (see Appendix 3) was added to all six Eppendorf tubes which were placed in ice, next 0.2 ml of enzyme substrate was added to the first three tubes (the actual assay tubes not the control tubes). The substrate was 50 mM p-nitrophenyl phosphate (Sigma) also called p-NPP and its molecular weight is 263.1. The p-NPP was in powder form and stored at -20˚C in the dark. Before each assay the 50 mM solution (p-NPP mixed in distilled H_2O) was freshly prepared and stored in a bottle wrapped in tin foil as p-NPP is light sensitive. It was then placed on ice or in the fridge (4˚C) until use. Following the addition of the substrate, 0.1 ml of the enzyme (the media) was added to all six tubes and mixed (using a vortex). These tubes were then removed from ice and placed in a 37˚C incubation room for two hours. It was established in preliminary experiments that these conditions were appropriate (see Appendix 4).

After two hours of incubation the tubes were immediately removed and placed back on ice to stop any further enzyme reaction. 0.2 ml of 50 mM p-NPP (the substrate) was then added to the control tubes. Now all six tubes had exactly the same contents. Finally 0.6ml of 1 M Na_2CO_3 (mw=105.99) was added to all six tubes. The tubes were then mixed and their absorbances were read at 410 nm using a spectrophotometer (Bio-Rad Smartspec™ Plus) to determine how much p-NPP had been hydrolysed by the APase present in the media.

The absorbance readings of the control tubes were then subtracted from the absorbance readings of the actual enzyme assays. From these three values the amount of acid phosphatase units present in the entire 120 ml of media (the 40 ml pooled together from the three jars in one treatment) were calculated. This produced three values of raw data on the amount of acid phosphatase units present in each 120 ml lot of media. See section 2.2.6 for how this data was then used.

2.2.5 Protein Determination Assays

Protein determination assays were carried out on all of the liquid media collected from the growth experiments. The method used to determine the quantity of soluble protein in the media was based on the method outlined by Marion Bradford in 1976. The protein assay used was not the standard assay (Bradford, 1976) but the micro-assay (Bradford, 1976) which was for very minute amounts of protein.

2.2.5.1 The Standard Curve

Bovine Serum Albumin (BSA) was used for the standard curves. The concentrations of BSA used for the standard curves were 0 µg/ml (the control which was distilled water because the base of the BSA stock solutions was distilled H_20), 5, 10, 20 and 25 µg/ml. 0.1 ml of the appropriate BSA solution plus 1 ml of Bradford Reagent (see Appendix 5) were mixed and the absorbance readings were read at 595 nm for the standard curves. Triplicate assays were carried out for each concentration of BSA, and then once all the

absorbances were read the control absorbances were subtracted off the other absorbances and the resulting values were averaged. The mean for each concentration was then plotted on a graph to generate the standard curve. A new standard curve was made every time an assay was done on a different day or using a different Bradford Reagent (i.e. a newly made one). If there was more than one assay done in one day using the same Bradford Reagent then only one standard curve was made for that day's assays. This assay did not need to be carried out on ice.

2.2.5.2 The Protein Determination of Media from the Growth Experiments

Each assay consisted of 0.1 ml of liquid media and 1 ml of Bradford Reagent. Triplicate assays were carried out for each treatment (so every jar of media was used) and the control assays were once again 0.1 ml of distilled water and 1 ml of Bradford Reagent. The media and Bradford Reagent were mixed immediately after being combined and then left for 5-10 minutes before their absorbances were read at 595 nm using a spectrophotometer (the spectrophotometer was always blanked using distilled H_20 before each assay). The absorbance values of the controls were then subtracted from the absorbance values of the assays containing the tissue culture media. These final values were then calculated into X μg/120 ml (using the standard curve along with calculations) to see how many micro-grams of soluble protein were in 120 ml of media (120 ml was the amount of media per treatment). This raw data was then analysed and used to help work out the effects that estrogen had (if any) on the growth of potato plants in liquid

tissue culture media. To see how this raw data was used refer to section 2.2.6 (Analysis of the Raw Data).

2.2.6 Analysis of the Raw Data

The raw data from the fresh and dry weights of the roots and shoots (from the first part of the growth experiment i.e. not the enzyme and protein assays) for each treatment were averaged. For example, the mean for the three fresh weights of the roots of the control plants in one experiment was obtained. This mean value was recorded as so many mg per three plants (because there were three potato plants in a tissue culture jar). The averages were then plotted on line graphs using Microsoft excel software. Standard errors were calculated and used as error bars on the graphs. The graphs were then scrutinised for important information i.e. trends, and general results. All the raw data was also run through one-way ANOVA (Statistix 8) (see introduction about one-way ANOVA). This was used to get the P-values, F values and results of the Tukey HSD All-Pairwise Comparisons Test for the data to make sure that there was no excessive variation in the data and that it was valid for statistical comparisons. All of the above was also done for all of the raw data from the acid phosphatase assays (i.e. the data of the amounts of acid phosphatase units in 120 ml of media) and the raw data from the protein determination assays (i.e. the amount of soluble protein per 120 ml of media).

All the data and results were then used to find out what effect if any, estrogen had on the growth of potato plants grown in liquid tissue culture media.

2.3 Materials and Methods for the Effects of Estrogen on Microtuberization of Potato Plants Grown in Liquid Tissue Culture Media

2.3.1 Plant Material

The nodal explants required for this section of experiments were prepared from previously grown stock plants (see 2.1.2).

2.3.2 Estrogen Preparation

The estrogen stock solutions were prepared in exactly the same way and using the same estrogens and concentrations of estrogens as described in section 2.2.2 of the materials and methods chapter.

2.3.3 Initiating Tuberization in Tissue Cultured Potato Plants

First, three nodal explants were grown per tissue culture jar for four weeks in 40 ml of liquid MS tissue culture media with 3 % sucrose. The growth room used was the same growth room used for all of the other experiments i.e. the conditions were a 24 hr photoperiod with a temperature of ~22˚C. At the end of four weeks the media were drained from the jars and replaced with 40 ml of autoclaved (so that it was sterile) liquid MS media with 8 % sucrose (all of this was done inside a sterile laminar flow cabinet to minimize contamination). Once the MS media with 3 % sucrose was replaced by media containing 8 % sucrose (Yoon, 2000), the tissue culture jars containing the potato plants

(with the 8 % sucrose MS medium) were then placed inside a drawer so that they were completely in the dark (Yoon, 2000). The temperature inside the drawer was ~22˚C. The plants were then left for eight weeks (though they were checked weekly) to grow tubers. The whole time required for the entire tuberization process equalled twelve weeks (four weeks growing the plants in the light with 3 % sucrose in the medium and eight weeks growing them in the dark with 8 % sucrose in the medium). The above methods were trialed in preliminary experiments and the results can be found in Appendix 6.

2.3.4 The Effects of Estrogen on Tuberization Experiments

The experiments to find out how estrogen affected the tuberization of potato plants grown in liquid tissue culture media were conducted in two different ways. The first way was having estrogen in the whole time (right from explants through to the end of the experiment). The second way was only adding estrogen when the media were changed.

2.3.4.1 Tuberization of Potato Plants with Media Containing Estrogen for the whole Twelve-Week Period

The concentrations of estrogen used were once again 0 mg/L (as the control), 0.1, 1 and 10 mg/L. The estrogens used were β-estradiol, estrone and estriol. 80 µl of estrogen stock solution (or 100 % ethanol for the control) was used per 40 ml of liquid tissue culture media.

Three nodal explants were placed in each 250 ml clear polycarbonate tissue culture jar containing 40 ml of autoclaved tissue culture media with 3 % sucrose. There were three replicates (jars) with nine explants altogether per treatment. So there were twelve jars (36 explants) for the entire experiment: three jars each for the control, 0.1, 1 and 10 mg/L of estrogen treatments. 80 µl of the appropriate solution was added to each labelled jar (using the same method as described in section 2.2.3) containing the three explants and 40 ml of 3% sucrose-supplemented MS media under sterile conditions. These potato plants were then grown for four weeks in a growth room at ~22˚C and with constant lighting (i.e. a 24 hr photoperiod). The jars containing the potato plants were placed randomly on the shelves so that no one treatment would benefit if one area of the shelf was more suitable for growth than another. The plants were checked weekly to make sure they were growing and not contaminated.

After four weeks of growth the media were drained from the tissue culture jars and then replaced with 40 ml of fresh autoclaved liquid MS media containing 8 % sucrose instead of 3 % sucrose. Just before the new media was added to the jars the 80 µl of correct estrogen solution (or 100 % ethanol for the control plants) was carefully squirted into 40 ml of the 8 % sucrose MS media and swirled around to evenly distribute it throughout the media.

After dispensing the new media (which still contained the same concentrations of estrogen as in previous media), all the twelve tissue culture jars containing the plants were placed in a drawer so that they were in complete darkness. They were placed

randomly in the drawer just in case a fraction of light managed to reach some jars. The temperature inside the drawer was ~22˚C. The plants were left for another eight weeks so that they had sufficient time to grow tubers. They were checked about every week.

After eight weeks the potato plants were removed from the dark and any microtubers that had grown were harvested. The number of microtubers per jar (three plants) was recorded. Each tuber was weighed and the weight was recorded. To get the fresh weight of all the tubers per jar, the individual weight of each tuber was added together. The tubers from each jar were wrapped in labelled tin foil (so all the tubers from one jar were wrapped in one piece of tin foil and no tubers from one jar were mixed with other ones from another jar). For the larger tubers, they were sliced up before being wrapped in the foil so that they would dry better. The tubers in the foil were then placed in an oven (Qualtex) at ~65˚C for approximately ten days to dry. Ten days were sufficient to dry all of the tubers. After the tubers were dried, their weight was recorded (they were taken out of the foil to be weighed). This was the dry weight of all of the tubers per three plants (not the individual weights). The data of the number of tubers and their fresh and dry weights was used (see section 2.3.5). This experiment was carried out twice using β-estradiol as the estrogen, twice using estrone and twice using estriol as the estrogen.

2.3.4.2 The Tuberization of Potato Plants with Media Containing Estrogen for only the last Eight Weeks of the Twelve-Week Period

This experiment was done exactly the same as the previously described experiment except the estrogen (and also the 100 % ethanol for the control) was not present during the first four weeks of growth. So while the potato plants were growing for four weeks in the MS media with 3 % sucrose there was no estrogen in the media and no ethanol in the control plants media. The estrogen was added at the same time as the 8 % sucrose-containing MS media was added to the four weeks old potato plants. (This was when the 3 % sucrose MS media was drained off and replaced by the 8 % sucrose-containing liquid MS media). From here on the experiment was identical to the previous experiment outlined in section 2.3.4.1. This experiment was carried out once using β-estradiol as the estrogen, once using estrone and once using estriol as the estrogen.

2.3.5 Analysing the Data from the Tuberization Experiments

The raw data from the number of tubers and the fresh (for all the tubers in one jar, not the individual tuber weights) and dry weights of the tubers from each jar for each treatment were averaged. The four resulting means (i.e. one mean for the control, one for 0.1 mg/L of estrone etc) were recorded as mg per three plants (because there were three plants in a tissue culture jar). The averages were then plotted on line graphs using Microsoft excel. Standard errors were calculated and were used as the error bars on the graphs. The graphs were then checked to see if there was any important information i.e. trends. All the raw

data was run through one-way ANOVA (Statistix 8) to get the P value, F value and results for the Tukey HSD All-Pairwise Comparisons Test to check that there was no excessive variation in the data and see if there were any trends.

The individual weights of the tubers were used to see what percentage of tubers from each treatment fell in the following weight categories: 0-50 mg, 50-100 mg, 100-500 mg and 500+ mg. These particular categories were chosen because there seemed to be a reasonable amount of tubers between these weight ranges. The percentages were displayed on column graphs. The results to this were checked to see if any one treatment seemed to have more tubers in one weight category than the other treatments.

All the data and results were then used to find out what effect if any estrogen had on the tuberization of potato plants grown with estrogen in their liquid tissue culture media.

3. Results

3.1 The Effects of Estrogen on the Growth of Potato Plants Grown in Liquid Tissue Culture Media: Quantitative Results

3.1.1 Dry Weight of the Roots and Shoots of the Potato Plants

The fresh weight data from the experiments follow the same pattern as the dry weight data. Therefore, all of the following results will be about the dry weights but the below observations also apply for the fresh weights of the potato plants. The results are from all of the eight experiments (the two growth experiments using β-estradiol, three using estrone and three using estriol as the estrogen i.e. see table two). There was no distinct difference in growth patterns between the three estrogens used (Figs 1-8).

The roots of the control plants had the highest dry weight twice out of the eight experiments (Figs. 1 and 8) and their shoots had the heaviest dry weight once (Fig. 8). The control plants produced the lowest weighing roots three times out of eight experiments (Figs. 2, 4 and 6) and the lowest weighing shoots four times out of eight experiments (Figs. 2-4 and 6).

The potato plants grown in media with 0.1 mg/L of estrogen had the highest dry weight of roots two times out of eight experiments (Figs. 3 and 7) and the highest dry weight of

shoots three times out of eight experiments (Figs. 1,3 and 7). The plants grown with 0.1 mg/L of estrogen had the lowest dry weight of roots and shoots once (Fig. 5).

Those potato plants grown with 1 mg/L of estrogen in their tissue culture media had the heaviest dry weight of roots and shoots once (Fig. 2) and they never had the lightest dry weight of roots and shoots.

The plants grown with 10 mg/L of estrogen in their medium weighed the most (both roots and shoots) three times out of eight experiments (Figs. 4-6). The potato plants' roots from the 10 mg/L of estrogen treatments weighed the least four times (50%) out of eight experiments (Figs. 1,3,7 and 8) and their shoots were the lightest three times out of eight experiments (Figs. 1,7 and 8).

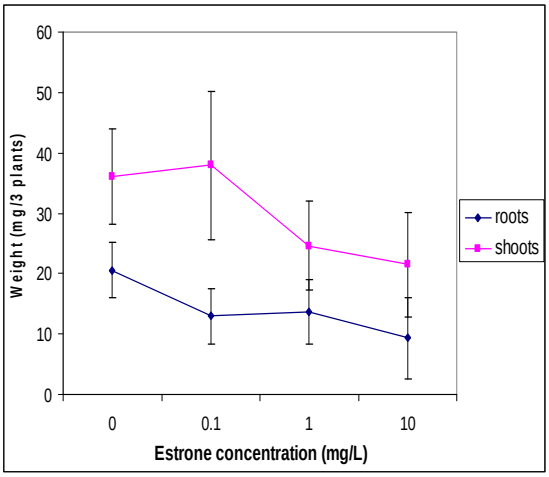


Figure 1. Dry weights of potato plants from the estrone trial run.

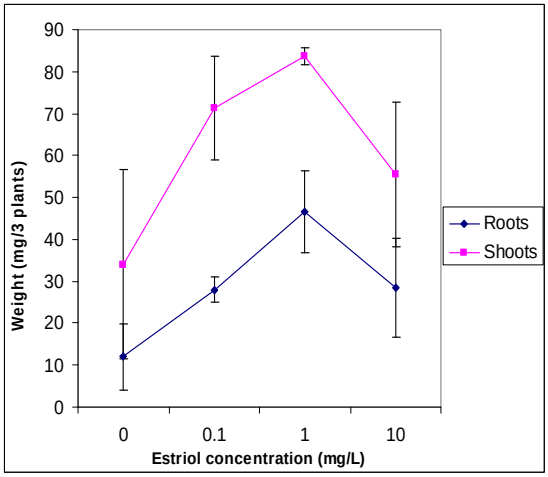


Figure 2. Dry weights of potato plants from the estriol trial run.

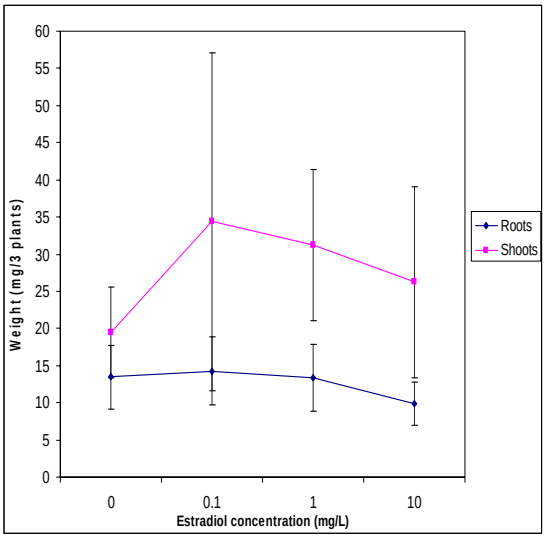


Figure 3. Dry weights of potato plants from the 1st estradiol growth experiment.

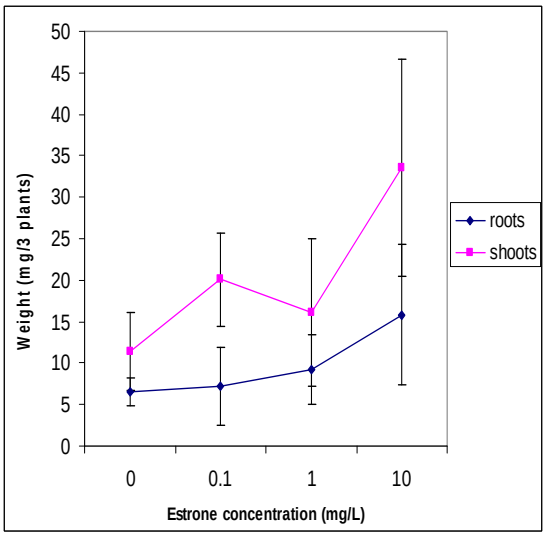


Figure 4. Dry weights of potato plants from the 1st non-trial run estrone growth experiment.

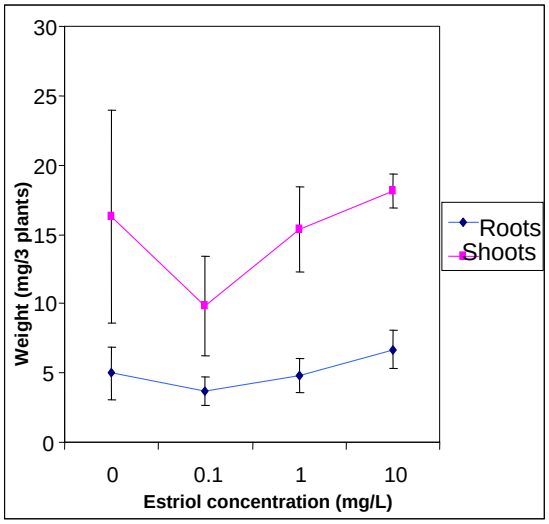


Figure 5. Dry weights of potato plants from the 1st non-trial estriol run.

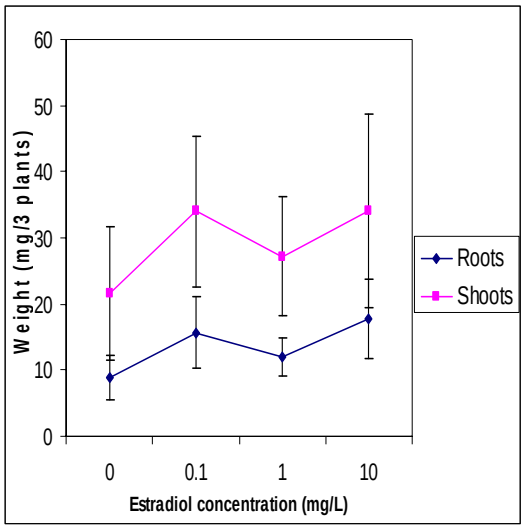


Figure 6. Dry weights of potato plants from the 2nd estradiol experiment.

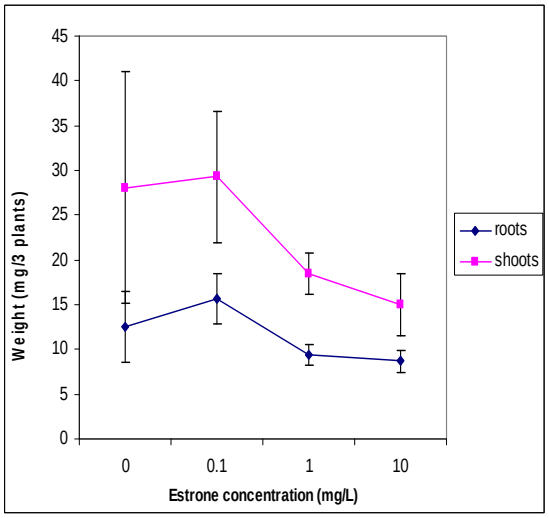


Figure 7. Dry weights of potato plants from the 2nd non-trial estrone run.

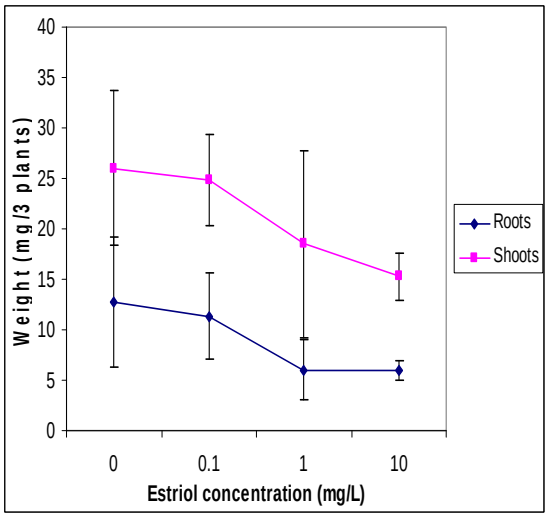


Figure 8. Dry weights of potato plants from the 2nd non-trial estriol run.

3.1.1.1 One-Way ANOVA Results for the Dry Weight of the Potato Plants' Roots and Shoots

Replicate (media type)	P value	F value (F max=142)	Tukey HSD Test. Does the data show a trend? Yes/No	Has the data passed the requirements for statistical comparison? Yes/No
Estrone trial run				
Roots	0.5413	2.1720	No	Yes
Shoots	0.5378	2.7478	No	Yes
Estriol trial run				
Roots	0.1238	15.162	No	Yes
Shoots	0.2104	124.56	No	Yes
1st Estradiol run				
Roots	0.8778	2.5933	No	Yes
Shoots	0.8905	13.924	No	Yes
1st Estrone non-trial				
Roots	0.6225	25.531	No	Yes
Shoots	0.3786	7.9376	No	Yes
1st Estriol non-trial				
Roots	0.5353	3.4362	No	Yes
Shoots	0.6223	39.851	No	Yes
2nd Estradiol run				
Roots	0.5511	4.4896	No	Yes
Shoots	0.8403	2.6198	No	Yes
2nd Estrone non-trial				
Roots	0.2775	11.473	No	Yes
Shoots	0.5087	31.924	No	Yes
2nd Estriol non-trial				
Roots	0.5745	39.841	No	Yes
Shoots	0.6221	15.459	No	Yes

Table 2. One-way ANOVA results for the dry weight of the potato plants' roots and shoots for all eight growth experiments. The data is ok if the P value is less than one and the F value is less than the F max value of 142.

3.1.2 Acid Phosphatase Activity in Culture Media

3.1.2.1 Media Containing β-estradiol (estradiol)

There were two independent experiments (replicates) done using estradiol as the estrogen so acid phosphatase assays were performed on media from both experiments. As the pattern shown in the data was the same for both experiments only one experiment will be displayed. Figure nine is shown here as an example of the typical set of data that were obtained.

The control media (without β-estradiol) from both experiments had the least amount of acid phosphatase. So the potatoes grown with no estrogen exuded the least amount of acid phosphatase into the liquid tissue culture medium. In both assays the media with the 10 mg/L of estradiol contained slightly more acid phosphatase than the control media, but in both cases the standard errors over-lapped. The media from the plants grown with 0.1 mg/L and 1 mg/L of estradiol both produced more acid phosphatase per 120 ml of media than the control and 10 mg/L of estradiol media. The highest production of acid phosphatase was from the plants grown with 1mg/L of estradiol (Fig. 9).

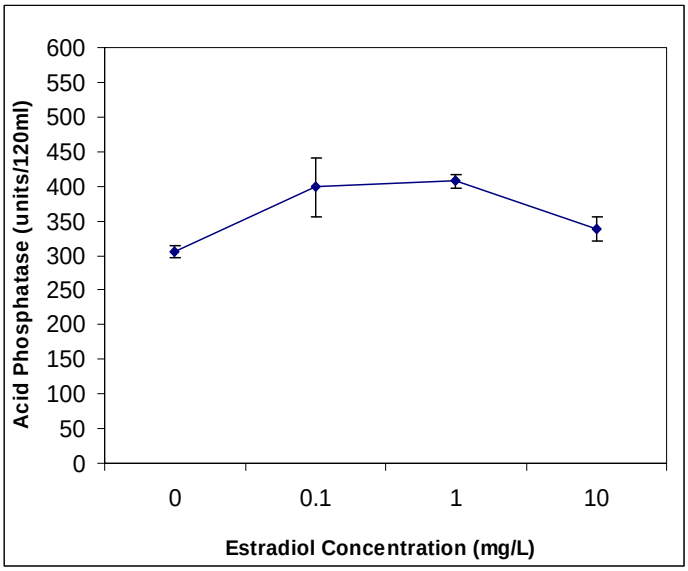


Figure 9. The acid phosphatase results for the 1st experiment containing β-estradiol as the estrogen.

3.1.2.2 Results for Media Containing Estrone

There were three experiments done using estrone as the estrogen and all three experiments produced similar results. However, there were slight differences. The graph (Fig. 10) used as an example is typical of the data obtained and is one of the graphs that has a nearly identical pattern with another one.

The plants grown with 0.1 mg/L and 10 mg/L of estrone exuded the least amount of acid phosphatase into their liquid media two times out of the three experiments. (In one experiment the 0.1 mg/L medium contained the most acid phosphatase but in that assay there was not much difference in acid phosphatase activity for all of the treatments). The control potato plants released slightly more acid phosphatase activity than the plants grown with 10 mg/L of estrone two out of three times. In the assay that showed the media containing 10 mg/L of estrone having more acid phosphatase activity than the control media, the difference was very slight. The media with the 1 mg/L of estrone had the highest level of acid phosphatase activity twice out of three experiments.

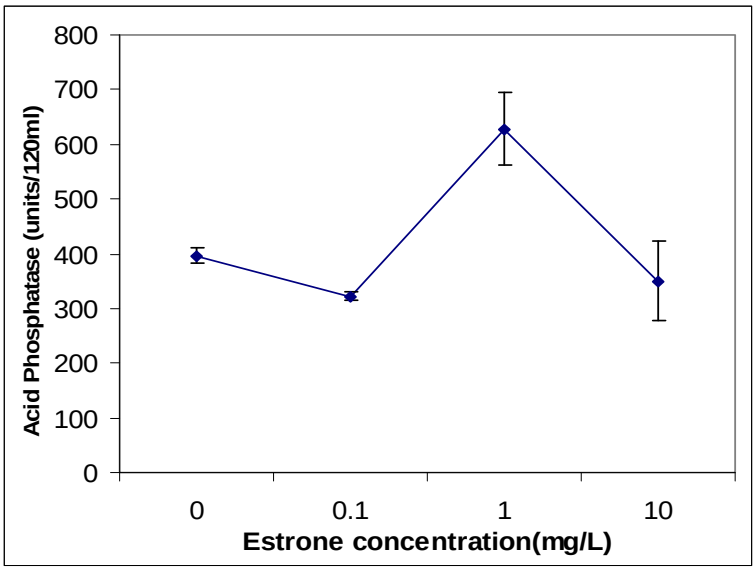


Figure 10. The acid phosphatase results for the 1st experiment containing estrone as the estrogen.

3.1.2.3 Results for Media Containing Estriol

All three experiments produced a similar pattern in the results of the acid phosphatase assays (see fig. 11 as a typical example).

In all three experiments the potato plants grown with 1 mg/L of estriol in the liquid tissue culture medium released the greatest amount of acid phosphatase activity per 120 ml of media. The control plants released the least amount of acid phosphatase activity two times out of the three experiments and only once released a fraction more acid

phosphatase than the plants grown with 10 mg/L of estriol. The plants grown in the media containing 0.1 mg/L and 10 mg/L of estriol released a very similar amount of acid phosphatase activity in all three experiments. Overall the media containing 1 mg/L contained the most acid phosphatase activity and the control media the least.

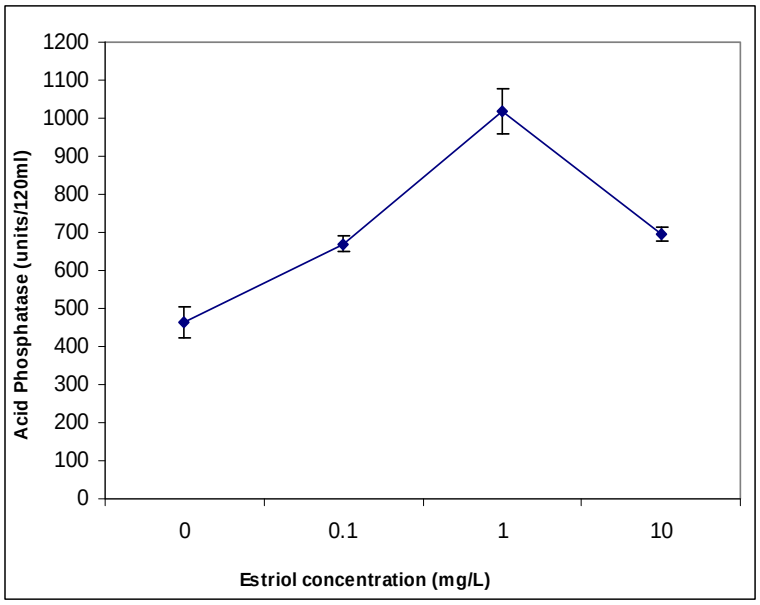


Figure 11. The acid phosphatase results for the trial run experiment containing estriol as the estrogen.

3.1.2.4 The Overall Results for the Acid Phosphatase Assays

For the assays done on the media containing β-estradiol and estriol, the control plants released the least amount of acid phosphatase (APase) activity. In the media containing estrone, the control plants released only slightly more APase activity than those grown with 0.1 mg/L of estrone (the media from this treatment also had the lowest level of APase activity for the experiments using estrone). The media containing 1 mg/L of all

three estrogens (estradiol, estrone and estriol) had the highest levels of APase activity per 120 ml of tissue culture media (Fig. 12).

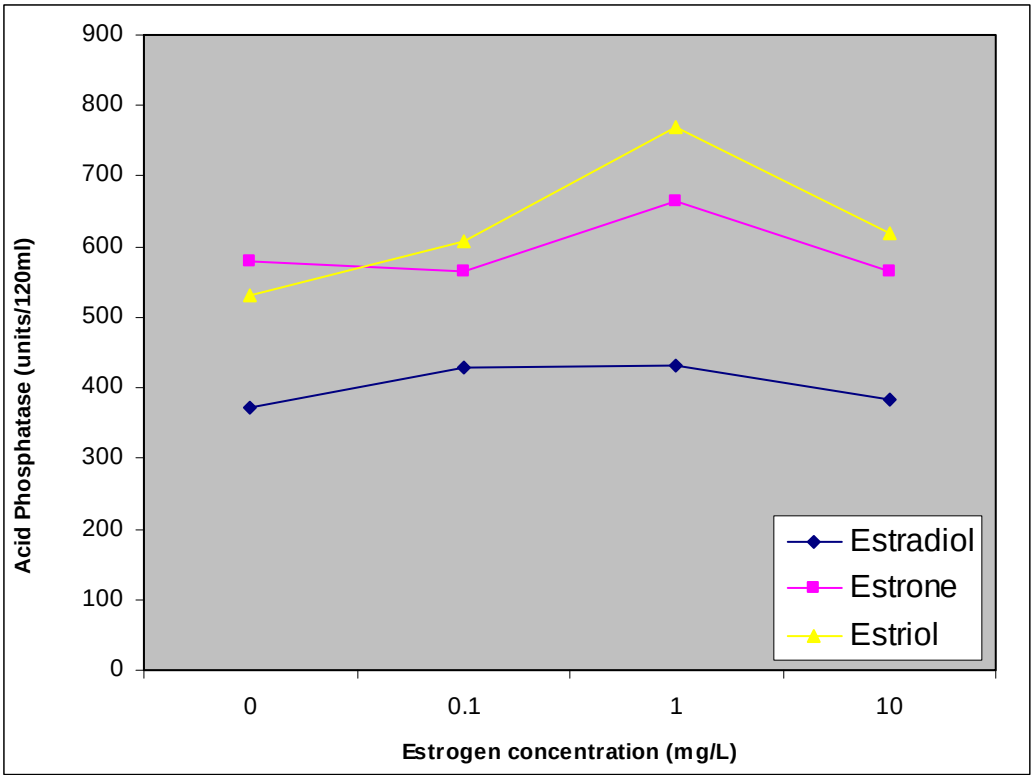


Figure 12. The overall results of the acid phosphatase assays. The plotted data for each estrogen is the average of the amount of acid phosphatase in 120 ml of liquid tissue culture media from each experiment for that particular estrogen.

3.1.2.5 One-Way ANOVA Results for the Data from the Acid Phosphatase Assays

Replicate (media type)	**P value**	**F value (F max=142)**	**Tukey HSD test. Does the data show a trend? Yes/No**	**Has the data passed the requirements for statistical comparison? Yes/No**
Estrone Trial	0.4799	19.803	No	Yes
Estriol Trial	0.0001	11.464	Yes	Yes
1st B-estradiol	0.0473	24.310	No	Yes
1st Estrone non-trial	0.0090	94.586	Yes	Yes
1st Estriol non-				

trial	0.2341	20.349	No	Yes
2nd B-estradiol	0.9692	127.79	No	Yes
2nd Estrone non-trial	0.6715	96.500	No	Yes
2nd Estriol non-trial	0.2960	16.972	No	Yes

Table 3. The One-way ANOVA results for the acid phosphatase assays for all eight growth experiments. The data is ok if the P value is less than one and the F value is less than the F max value of 142.

3.1.3 Protein Determination Assay Results

There are no distinct differences among the three estrogen treatments in the amount of soluble protein per 120 ml of tissue culture media. The results are not uniform and the variation in patterns in the data is the same for all of the three estrogens so all findings are combined and presented from the entire eight experiments.

Four out of eight of the experiments showed a statistical trend in the amount of soluble protein in the tissue culture media (to see the one-way ANOVA results refer to table 4). These four experiments are: the 1st and 2nd growth experiment using β-estradiol as the estrogen, the 2nd non-trial run of estrone and the 1st non-trial run using estriol (see figs.15 and 17-19). Three out four of these experiments that showed a trend using one-way ANOVA follow a very similar pattern. They are the: 1st growth experiment using β-estradiol as the estrogen, the 2nd non-trial run of estrone and the 1st non-trial run using estriol (see figs. 15,17 and 19). In these three experiments, the highest soluble protein content was found in the media after four weeks growth from the treatments of 1 mg/L of estrogen. The media from the 10 mg/L of estrogen and the control treatments always

contained much less soluble protein than the media from the 1 mg/L of estrogen treatments.

Out of the entire eight experiments the media from the control treatments contained the most amount of soluble protein once (Fig. 14) and the least twice (Figs. 15 and 20). The control media contained more protein than those from the treatment of 0.1 mg/L of estrogen three times, 1 mg/L of estrogen twice and 10 mg/L of estrogen five times out of the eight experiments.

Out of all of the eight experiments the treatment of 0.1 mg/L of any given estrogen contained the greatest amount of protein per 120 ml of tissue culture media twice (Figs. 16 and 18) and the least once (see fig. 13). The media from the treatments of 0.1 mg/L of estrogen contained more soluble protein than the control media five times out of eight (which is 62.5%), more than those of the treatment of 1 mg/L of estrogen four times (50%) and 10 mg/L of estrogen five out eight times (62.5 %).

From all of the eight experiments the media from the 1 mg/L of estrogen treatments contained the greatest amount of soluble protein three out of eight times (Figs. 15, 17and 19) and the least once (Fig. 16). These media from the 1 mg/L of estrogen treatments had more protein than the control media six out eight times (75%), more than the treatments of 0.1 mg/L of estrogen four out eight times (50%) and 10 mg/L of estrogen five out of eight times (62.5%).

Two times out of the entire eight experiments the media from the treatments of 10 mg/L of estrogen contained the highest amount of soluble protein (Figs. 13 and 20) and the lowest amount of protein in four out of eight experiments which is 50% (Figs. 14 and 17-19). The 10 mg/L of estrogen treatments resulted in more protein than the other three treatments three times out of the eight experiments (not all in the same experiments though).

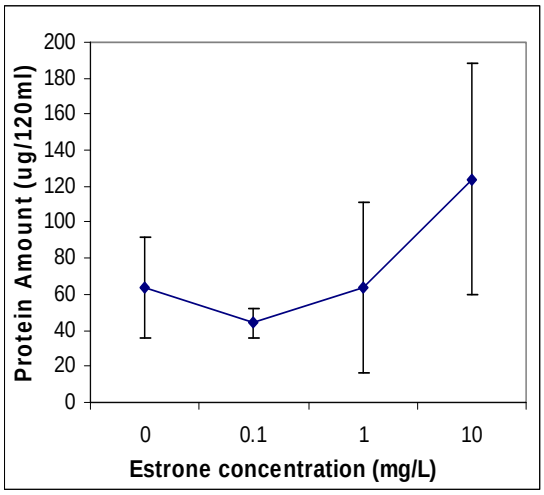


Figure 13. The amount of protein (µg) produced by the plants per 120 ml of media for each treatment in the estrone trial run.

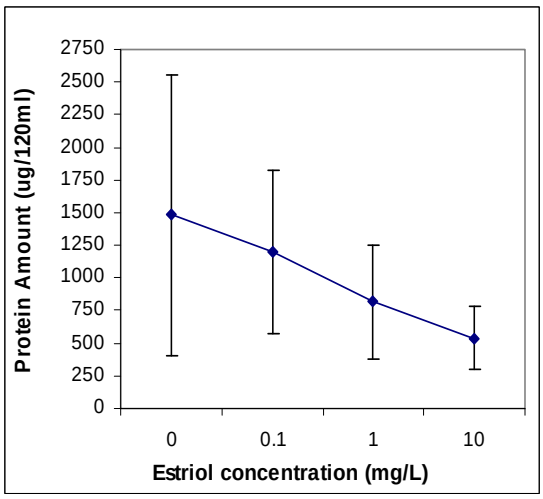


Figure 14. The amount of protein (µg) produced by the plants per 120 ml of media for each treatment in the estriol trial run.

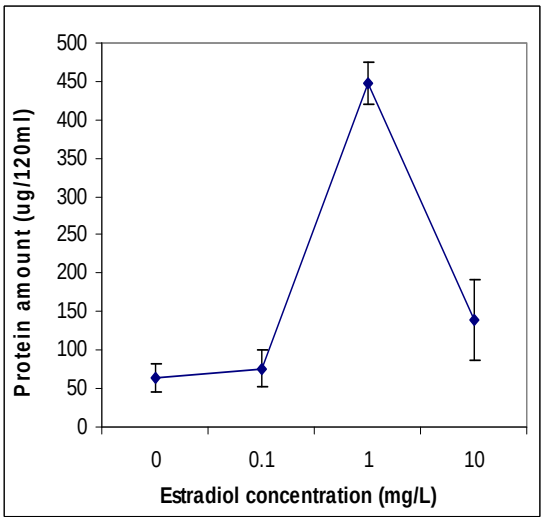


Figure 15. The amount of protein (µg)

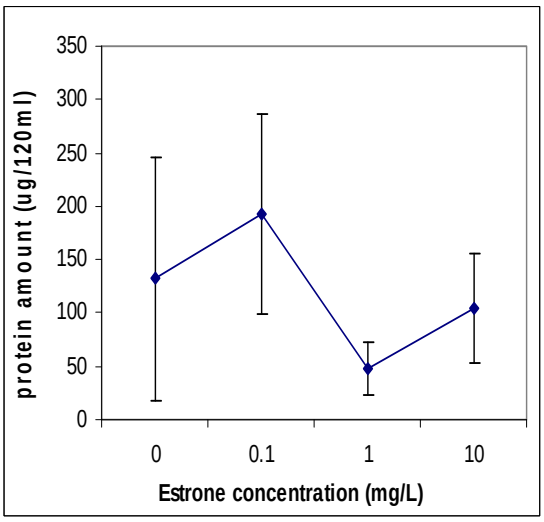


Figure 16. The amount of protein (µg)

produced by the potato plants per 120ml of media for each treatment in the 1st β-estradiol run.

produced by the potato plants per 120ml of media for each treatment in the 1st non-trial estrone run.

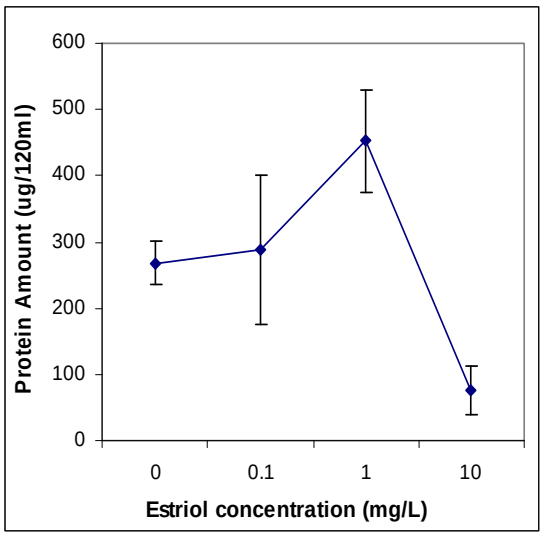


Figure 17. The amount of protein (µg) produced by the potato plants per 120 ml of media for each treatment in the 1st non-trial estriol run.

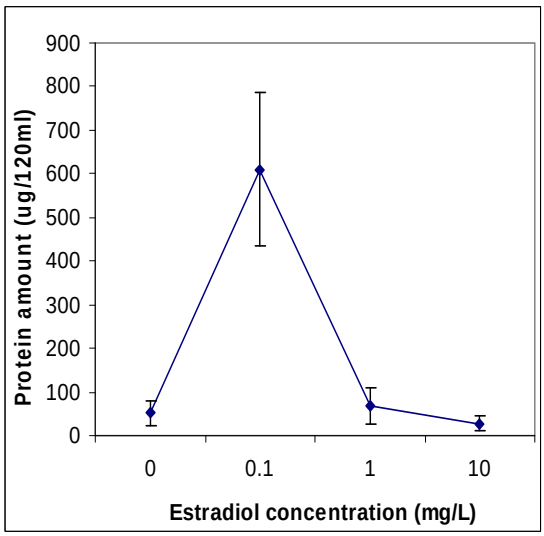


Figure 18. The amount of protein (µg) produced by the potato plants per 120ml of media for each treatment in the 2nd estradiol run.

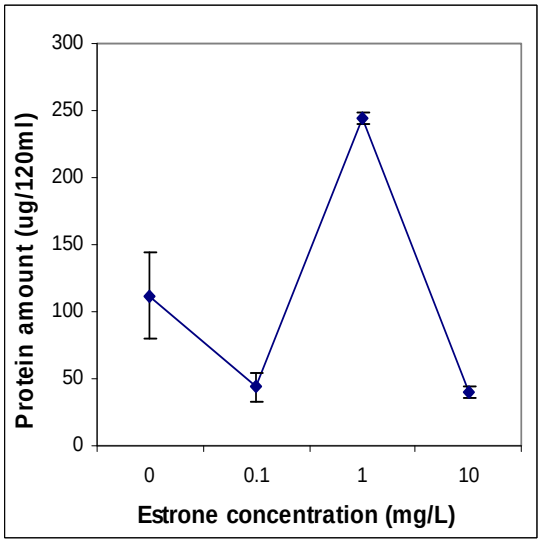


Figure 19. The amount of protein (µg)

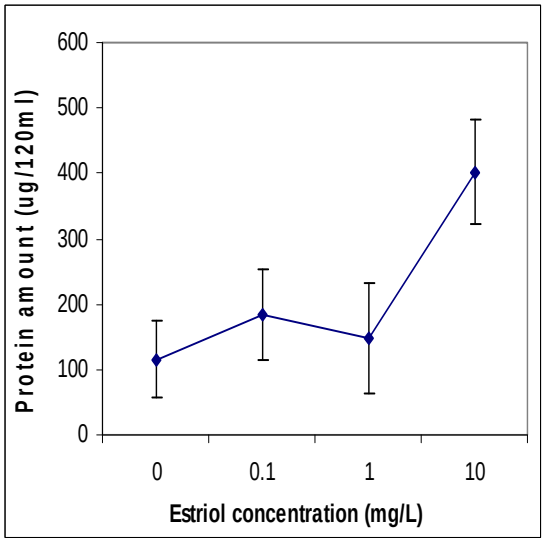


Figure 20. The amount of protein (µg)

produced by the potato plants per 120 ml of media for each treatment in the 2nd non-trial estrone run.

produced by the potato plants per 120 ml of media for each treatment in the 2nd non-trial estriol run.

3.1.3.1 The One-Way ANOVA Results

For four of the eight experiments, the data from the protein determination assays showed a trend according to the one-way ANOVA.

Replicate (media type)	**P value**	**F value (Fmax=142)**	**Tukey HSD test. Does the data show a trend? Yes/No**	**Has the data passed the requirements for statistical comparison? Yes/No**
Estrone trial	0.5965	64.750	No	Yes
Estriol trial	0.7676	20.028	No	Yes

1st B-estradiol	0.0001	9.2105	Yes	Yes
1st Estrone non-trial	0.6495	21.000	No	Yes
1st Estriol non-trial	0.0400	12.000	Yes	Yes
2nd B-estradiol	0.0053	101.43	Yes	Yes
2nd Estrone non-trial	0.0001	64.000	Yes	Yes
2nd Estriol non-trial	0.0909	2.1971	No	Yes

Table 4. The One-way ANOVA results for the protein determination assays for all eight growth experiments. The data is ok if the P value is less than one and the F value is less than the F max value of 142.

3.1.4 Qualitative Results

These results are from overall observations of all replicates. About 75-85% of potato plants grown follow the general trends/observations indicated below.

Visual Observations of Potatoes after Four Weeks of Growth:

The differences between treatments started to appear between about week one and week two of growth.

β-estradiol Control (0 mg/L): The potato plants were generally well formed. They had a good amount of leaves and roots. The leaves, stems and roots were well formed and

appeared to be healthy. There was an average amount of hairs on the stems (Plate 4).The amount of hairs on the stems varied quite a lot between individual plants.

Plate 4. An example of potato plants from the B-estradiol control.

β-estradiol 0.1 mg/L: The plants were generally of a similar size to the control . The leaves and roots were healthy looking. They had plenty of roots and about the same amount as the controls. They had a moderate amount of hairs on the stems. The amount of leaves, stems and roots were similar to the control plants. (Plate 5).

Plate 5. An example of potato plants from the B-estradiol 0.1 mg/L treatment.

β-estradiol 1 mg/L: The plants were about the same size as the control. The stems and leaves were healthy looking. The stems were about as hairy as the control. Some of the roots were clumpy looking and were not as long as the roots on the control plants. Some of the roots looked stunted. Other than the difference in the roots the plants of this treatment appeared similar to the plants of the control. (Plate 6).

Plate 6. An example of potato plants from the B-estradiol 1 mg/L treatment.

β-estradiol 10 mg/L: The plants did not look as healthy as the control plants. Deformities were found in the stems, leaves and roots of the potato plants. The lower parts of the stems were thickened and less defined than those of the control plants. Some of the leaves were quite small compared with others and appeared to be deformed. The roots were very clumpy looking. Root hairs were very short and were thicker than those of the control'. The top of the roots and the bottom of the stems were apparently covered with small whitish lumps of callus tissues (Plate 7) (Next page).

Plate 7. An example of potato plants from the B-estradiol 10 mg/L treatment.

Estrone control (0 mg/L): The plants were very healthy looking. There were plenty of healthy, well formed leaves. The stems were well formed and some were quite hairy. There were plenty of long, healthy looking roots and some aerial roots. These aerial roots grew on some plants and not on others and there did not appear to be a distinct pattern to their appearance. (Plate 8).

Plate 8. An example of potato plants from the estrone control.

Estrone 0.1 mg/L: Some plants in this treatment were a little stunted compared to the control plants, but overall the stems and leaves appeared as healthy as those of the control. The stems might not be quite as hairy as the control. The roots appeared thicker,

shorter and less abundant than those of the control plants. Overall, however, the roots still appeared reasonably healthy (Plate 9).

Plate 9. An example of potato plants from the estrone 0.1 mg/L treatment.

Estrone 1 mg/L: The leaves of the potato plants appeared to be slightly deformed. They were not as abundant as in the control. Once again some of the stems were as hairy as those of the control but others were not. Some of the stems were thick and slightly deformed and occasionally looked like they were slightly callused. The roots still looked quite healthy but they were thickened compared to those of the control. Also the roots were shorter than and not as profuse as those of the control plants (Plate 10) (Next page).

Plate 10. An example of potato plants from the estrone 1 mg/L treatment.

Estrone 10 mg/L: Overall the plants did not look as healthy as the control plants although a few plants still looked fairly healthy. The leaves were not highly abundant though they were still present. Some of the leaves looked deformed. The tips of the stems looked reasonably healthy, but down lower near the roots they looked deformed and showed callus-like growth. Sometimes a whitish callus-like powder might appear on the lower stems and upper roots. The roots were usually very shrunken looking and in some cases they were almost non-existent. Some roots were stumpy and looked almost like small stems (Plate 11) (Next page).

Plate 11. An example of potato plants from the estrone 10 mg/L treatment.

Estriol control (0 mg/L): The potato plants looked very healthy. There were quite a few healthy looking leaves of various sizes. The stems looked healthy and some plants had grown aerial roots. The stems were moderately hairy. The roots were long and healthy, and were quite abundant (Plate 12).

Plate 12. An example of potato plants from the estriol control.

Estriol 0.1 mg/L: The potato plants had large healthy leaves. The stems were healthy, though they possibly might not be as hairy as the stems on the control plants. Some of the roots looked stumpy and they were shorter and thicker than those of the control. It appears that these potato plants had not grown as many roots as the controls. Other than the roots the plants still looked very healthy overall. In fact, some can grow larger than the control plants (Plate 13).

Plate 13. An example of potato plants from the estriol 0.1 mg/L treatment.

Estriol 1 mg/L: The potato plants were usually smaller than the control plants (in some cases however the plants had grown to be a slightly bigger than the controls). The leaves were not as abundant as those of the control but were still fairly healthy looking. The stems were about as hairy as those of the control and they were healthy looking. The roots were short and stunted. The top of the roots were thicker than the top of those of the control. There were not as many roots as in the control and a few of them looked similar

to stems (they were as thick as stems and were a greenish colour like the stems) (Plate 14).

Plate 14. An example of potato plants from the estriol 1 mg/L treatment.

Estriol 10 mg/L: Quite often the plants looked very deformed though some plants had grown rather well except for their roots. The leaves were usually small and looked thickened compared to the leaves on the control plants. The plants were usually much shorter than the control plants but the roots and stems looked thicker (i.e. they did not look as supple). The lower parts of the stems as well as the tops of the roots appeared to have formed some tiny whitish callus tissue. Most of the roots were very short and stumpy, and looked deformed. The plants of this treatment also had fewer roots than the control plants (Plate 15) (Next page).

Plate 15. An example of potato plants from the estriol 10 mg/L treatment.

In summary: As is to be expected the control plants from all experiments were very similar. Almost all control plants grown had healthy root systems. The overall pattern for all plants grown with β-estradiol, estrone and estriol is that even though at the lower concentrations (0.1 mg/L and 1 mg/L) the stems and leaves might look healthy, the roots were not as healthy as those of the control. With all three concentrations of estrogen and all three types of estrogen, the roots were almost always less healthy than those of the control potato plants.

3.2 Results from the Tuberization Experiments

3.2.1 Quantitative Results

3.2.1.1 Number of Tubers Formed

There were no outstanding differences in patterns from the data for the average number of tubers (per three plants) from each treatment of estrogen added at the beginning of the twelve week span or added only for the last eight weeks of the experiment. There was also no real difference in patterns among the three types of estrogen used. Therefore the results of all nine experiments carried out are combined and presented here.

The only noticeable difference between the potato plants grown with estrogen for the whole twelve weeks of the experiment and those grown with estrogen added at the beginning of the eight week stage was that on average the plants grown with estrogen added at the eight week stage produced less tubers than the plants grown with estrogen for the entire twelve weeks. This was also observed in the control plants however. Thus it cannot be linked solely to the addition of estrogen. This will be discussed further in the discussion.

The graphs the following observations refer to are figures 21-29.

In five out of nine experiments the control potato plants produced the most tubers per three plants (Figs. 21-23,25 and 26), which means that 55.56% of the time the control

plants grew the highest average amount of tubers (more tubers than the other three treatments). Only twice (22.22%) out of the nine experiments did the control plants produce less tubers on average than the plants treated with 10 mg/L of estrogen, which is the highest concentration of estrogen tested ; this was in the 2nd β-estradiol experiment (Fig. 24) and the 3rd estriol experiment (Fig. 29). Only once out of the nine experiments (11.11%) did the control plants produce the lowest average of tubers per three plants (Fig. 24). Six out of nine times (66.67%) (not all in the same experiments) the control plants produced more tubers per three plants than the potato plants treated with 0.1 mg/L and 1 mg/L of estrogen.

The potato plants grown with 0.1 mg/L of estrogen never produced the least average amount of tubers per three plants; but only once did they produce the highest average amount of tubers (Fig. 27). Six out of nine times (66.67%) the potato plants treated with 0.1 mg/L of estrogen produced more tubers than the potato plants treated with 1 mg/L of estrogen (Figs. 21-23, 25 ,27 and 29).

The plants treated with 1 mg/L of estrogen produced the least amount of tubers four times out of the nine experiments (Figs. 21, 23,25 and 29) which is 44.44% and only produced the most tubers twice (Fig. 24 and 28). However, five times out of the nine experiments (55.56%) those potato plants treated with 1 mg/L of estrogen produced on average more tubers than those potato plants treated with 10 mg/L of estrogen (Figs. 22, 24,26-28).

Only one time out of the whole nine experiments (11.11%) did the potato plants treated with 10 mg/L of estrogen produced the highest average of tubers per three plants (Fig. 29). Four times (44.44%) they produced the least amount of tubers (Figs.22 and 26-28).

Thus in six out of nine experiments (66.67%) the control plants and the potato plants treated with 0.1 mg/L of estrogen (which is the lowest concentration of estrogen tested), produced the most amount of tubers per three plants. Eight times out of nine experiments which is 88.89%, the potato plants treated with the two highest concentrations of estrogen (1 mg/L and 10 mg/L) produced the lowest mean of tubers (Figs. 21-23 and 25-29).

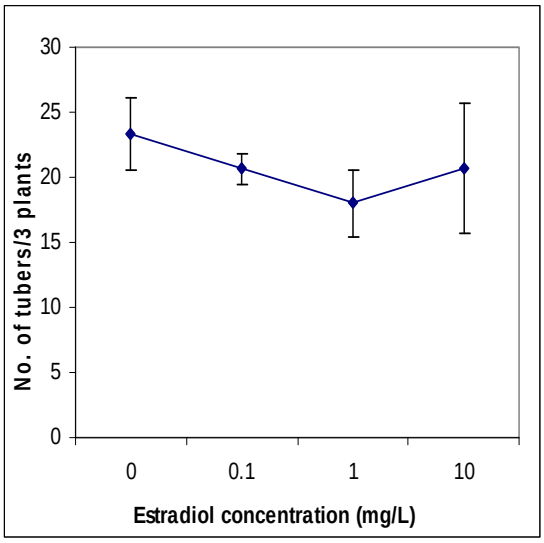


Figure 21. The mean number of tubers per 3 plants for the 1st β-estradiol tuberization tuberization experiment. This experiment had the estradiol added from the start.

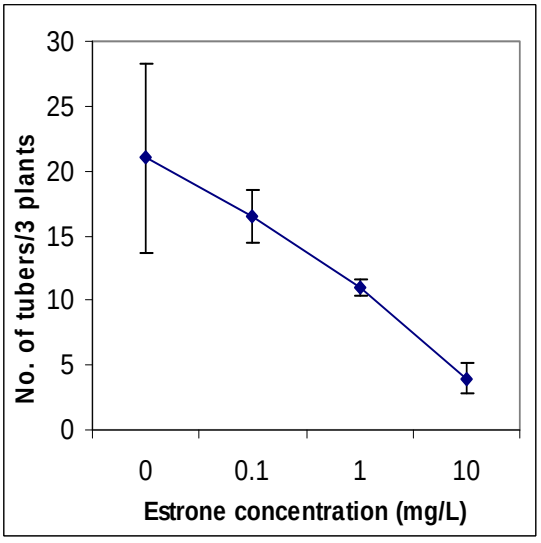


Figure 22. The mean number of tubers per 3 plants for the 1st estrone tuberization experiment. This experiment had the estrone added from the start.

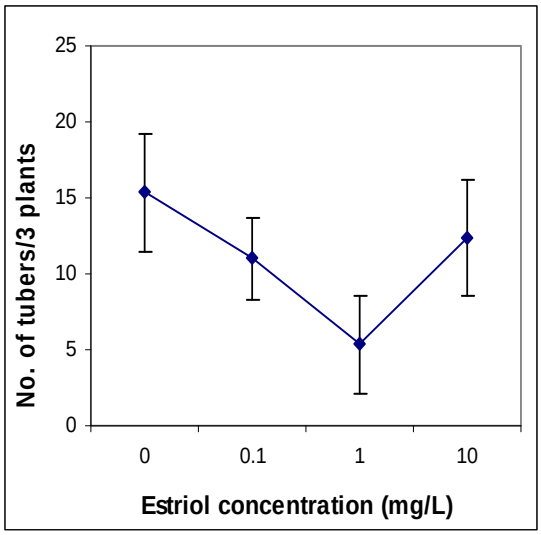


Figure 23. The mean number of tubers per 3 plants for the 1st estriol tuberization experiment. This experiment had the estriol added from the start.

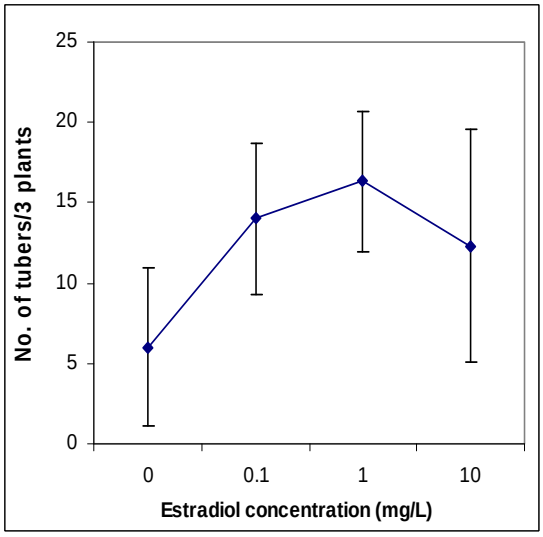


Figure 24. The mean number of tubers per 3 plants for the 2nd estradiol tuberization experiment. This experiment had the estradiol added from the start.

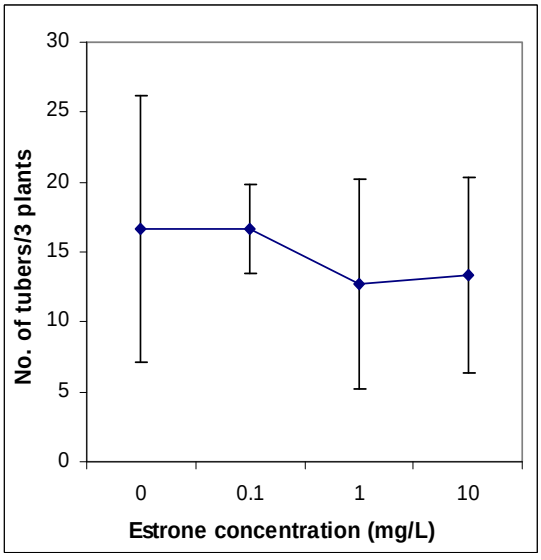


Figure 25. The mean number of tubers per 3 plants for the 2nd estrone tuberization experiment. This experiment had the estrone added from the start.

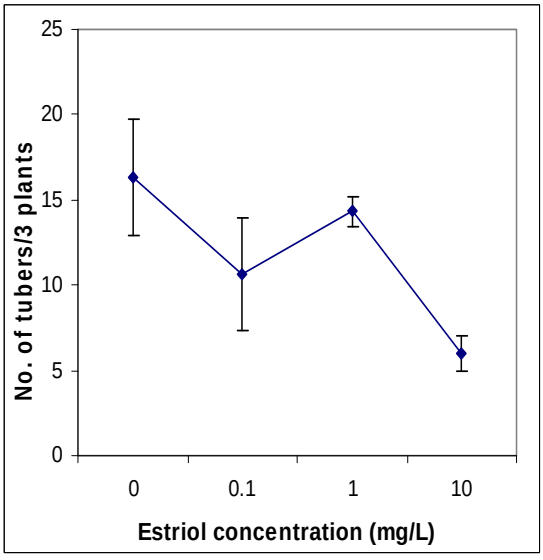


Figure 26. The mean number of tubers per 3 plants for the 2nd estriol tuberization experiment. This experiment had the estriol added from the start.

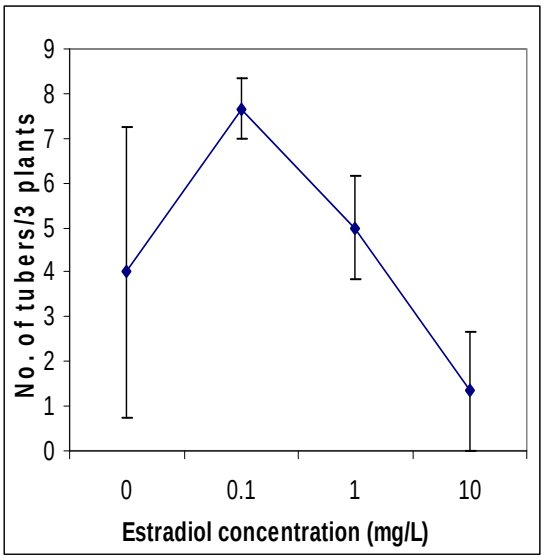


Figure 27. The mean number of tubers per 3 plants for the 3rd β-estradiol tuberization experiment. This experiment had the estradiol added at the 8 week stage (which starts after 4 weeks of growth).

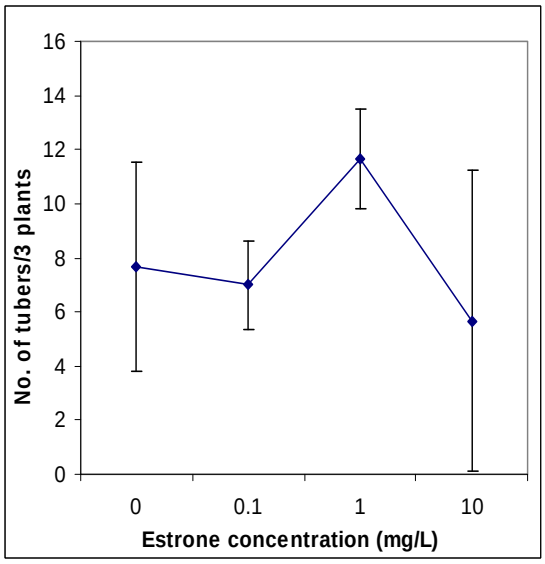


Figure 28. The mean number of tubers per 3 plants for the 3rd estrone tuberization experiment. This experiment had the estrone added at the 8 week stage (which starts after 4 weeks growth).

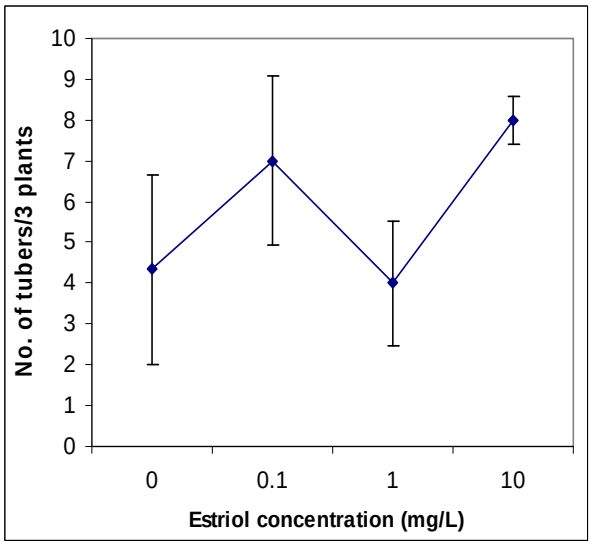


Figure 29. The mean number of tubers per 3 plants for the 3rd estriol tuberization experiment. In this experiment estriol was added at the 8 week stage (which is after 4 weeks growth).

3.2.1.1.1 The One-Way ANOVA Results

Replicate (media type)	P value	F value (Fmax=142)	Tukey HSD Test. Does the data show a trend? Yes/No	Has the data passed all requirements for statistical comparison Yes/No
1st β-estradiol	0.7089	17.154	No	Yes
1st Estrone	0.0164	50.480	Yes	Yes
1st Estriol	0.2858	2.1111	No	Yes
2nd β-estradiol	0.6960	2.7267	No	Yes
2nd Estrone	0.9643	8.9780	No	Yes
2nd Estriol	0.0722	14.714	No	Yes
3rd β-estradiol	0.1272	24.000	No	Yes
3rd Estrone	0.7294	12.042	No	Yes
3rd Estriol	0.3544	16.333	No	Yes

Table 5. The One-way ANOVA results for the number of tubers for all nine tuberization experiments. The first six experiments (replicates) had the estrogen with the plants for the plants' entire twelve weeks of growth. The last three replicates had the estrogen added at the eight week stage which came after four weeks of growth. The data is ok if the P value is less than one and the F value is less than the F max value of 142.

3.2.1.2 Results for Dry Weight of Tubers

The fresh weights of the tubers follow a very similar pattern to the dry weights, so only the dry weight results are presented here. There are no outstanding differences between results for the three estrogens and no outstanding differences between the two different types of tuberization experiments. Therefore all nine experimental results are combined and presented here.

The tubers formed by the control were the heaviest in three of the nine experiments (Figs. 30, 32 and 37). They were the lightest only once (Fig. 34). The mean dry weights of the tubers in the control were higher than those of the tubers from the 0.1 mg/L of estrogen treatments five out nine times (Figs. 30, 32, and 35-37) and those of the tubers from the 1 mg/L of estrogen six out of nine times (Figs. 30-33, 37 and 38). Seven times out of the nine experiments the control plants' tubers weighed more than those of the plants grown with 10 mg/L of estrogen (Figs. 30-33 and 35-37).

The dry weights of the tubers from the plants treated with 0.1 mg/L of estrogen were the heaviest twice (Figs. 31 and 33) and the lowest once (Fig. 37). Four times out of nine the dry weights of the tubers of this treatment were heavier than those of the tubers from the control plants (Figs. 31, 33, 34 and 38). Five times out of the nine experiments the average dry weights of the tubers from the 0.1 mg/L of estrogen treatments weighed more than the tubers from the 1 mg/L treatments (Figs. 30-33 and 38) and the 10 mg/L of estrogen treated potato plants (Figs. 30-33 and 36).

The dry weights of the tubers from the plants treated with 1 mg/L of estrogen were the heaviest twice (Figs. 35 and 36) and the lightest three times out of nine (Figs. 32, 33 and 38). The average weights of the tubers from plants treated with 1 mg/L of estrogen were heavier than those of the control tubers three out of nine times (Figs. 34-36), those of the tubers from the 0.1 mg/L of estrogen treatments four out of nine experiments and 10 mg/L of estrogen five times out of nine (Figs. 30, 31 and 35-37).

The tubers from the potato plants treated with 10 mg/L of estrogen had the highest dry weight twice (Figs. 34 and 38) and the lowest dry weight four times out of the nine experiments (Figs. 30, 31, 35 and 36). The tubers from this treatment had higher mean dry weights than the control tubers twice (Figs. 34 and 38) and they were heavier than those from the 0.1 mg/L and 1 mg/L of estrogen treatments four out of nine times (Figs. 32, 34, 37 and 38, and figs. 32-34 and 38 respectively).

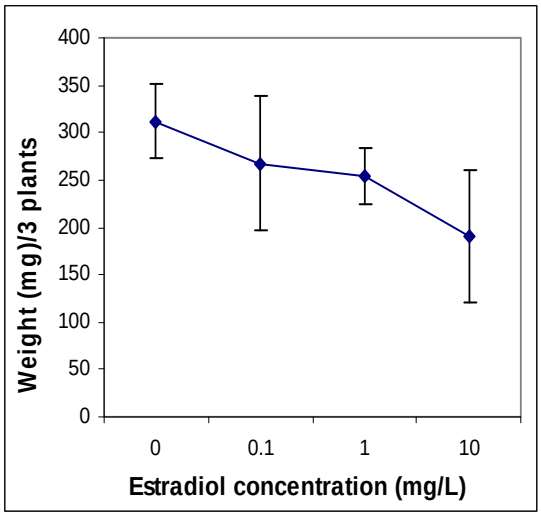


Figure 30. The average dry weight of tubers per 3 plants from the 1st β-estradiol experiment.

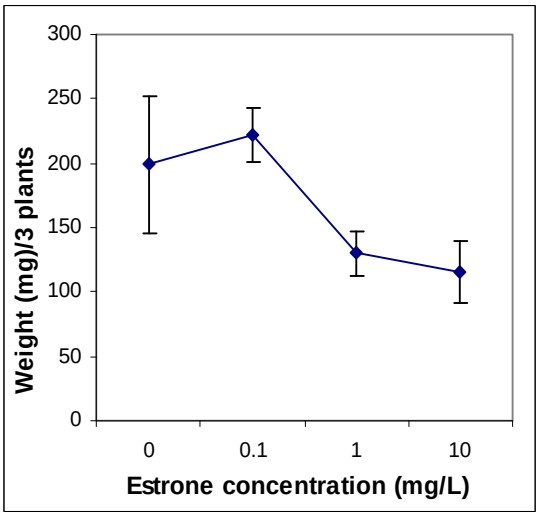


Figure 31. The average dry weight of tubers per 3 plants from the 1st estrone experiment.

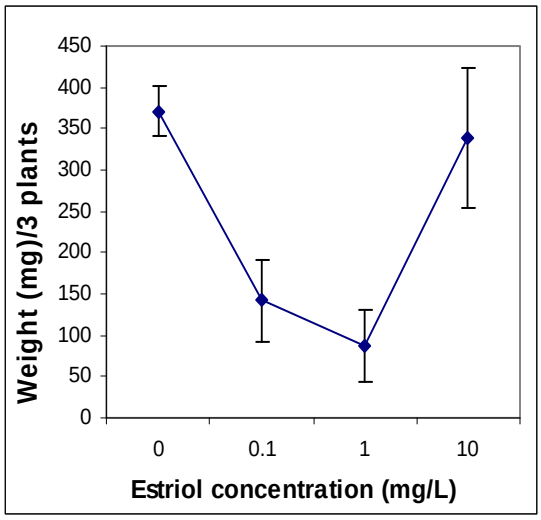


Figure 32. The average dry weight of tubers per 3 plants from the 1st estriol experiment.

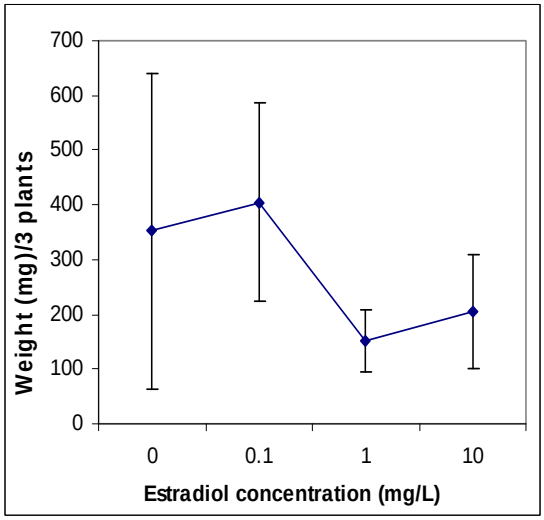


Figure 33. The average dry weight of tubers per 3 plants from the 2nd estradiol experiment.

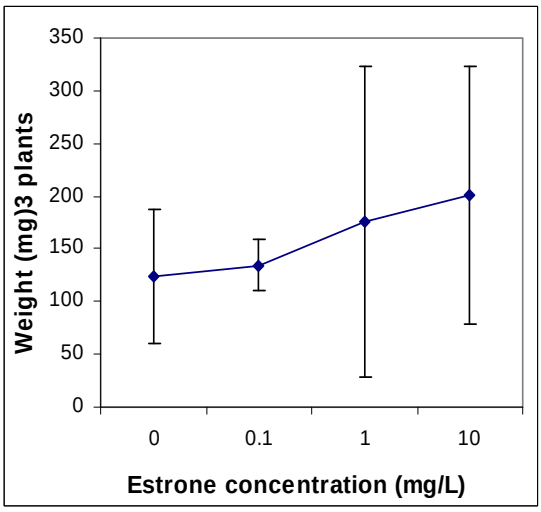


Figure 34. The average dry weight of tubers per 3 plants from the 2nd estrone experiment.

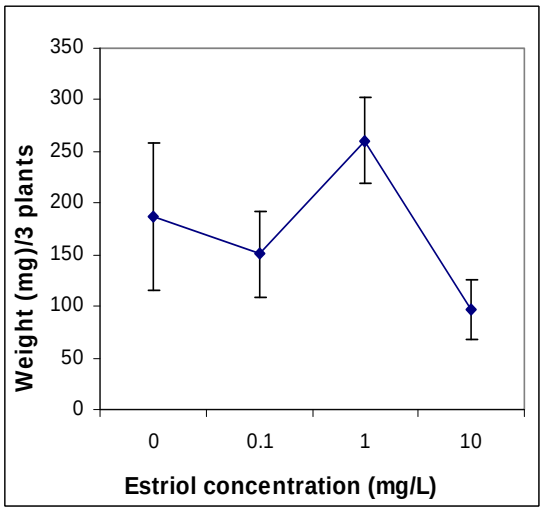


Figure 35. The average dry weight of tubers per 3 plants from the 2nd estriol experiment.

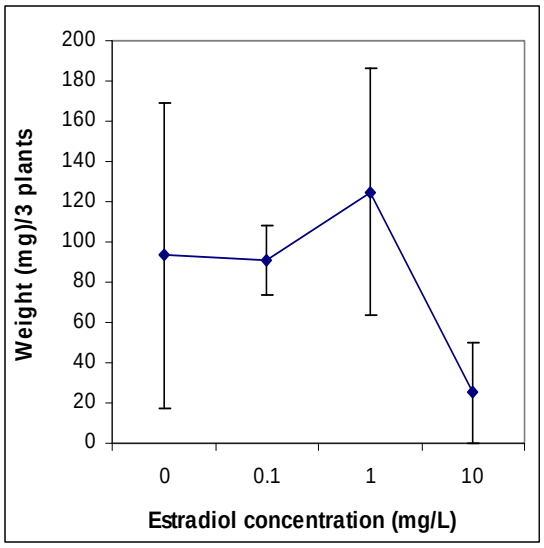


Figure 36. The average dry weight of tubers per 3 plants from the 3rd β-estradiol experiment.

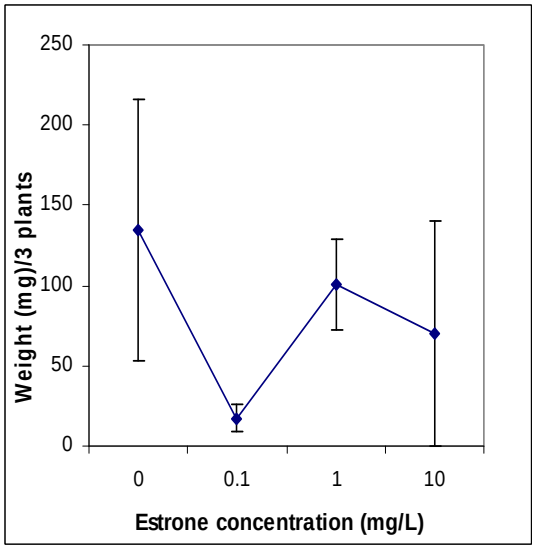


Figure 37. The average dry weight of tubers per 3 plants from the 3rd estrone experiment.

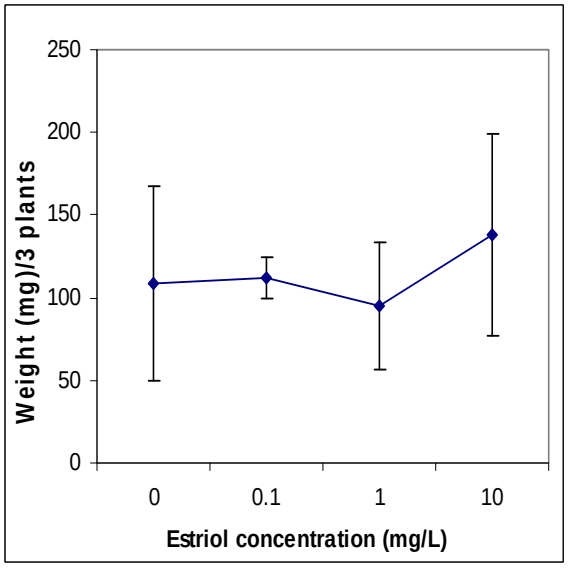


Figure 38. The average dry weight of tubers per 3 plants for the 3rd estriol experiment.

3.2.1.2.1 One-Way ANOVA Results

Replicate (Media type)	P value	F value (Fmax=142)	Tukey HSD Test. Does the data show a trend? Yes/No	Has the data passed the requirements for statistical comparison? Yes/No
1st β-estradiol	0.5199	5.4370	No	Yes
1st Estrone	0.1511	9.4405	No	Yes
1st Estriol	0.0153	7.5894	Yes	Yes
2nd β-estradiol	0.6719	26.035	No	Yes
2nd Estrone	0.9434	34.956	No	Yes
2nd Estriol	0.1957	6.1481	No	Yes
3rd β-estradiol	0.5322	19.342	No	Yes
3rd Estrone	0.6598	86.231	No	Yes
3rd Estriol	0.9302	25.301	No	Yes

Table 6. The One-way ANOVA results for the dry weight of tubers for all nine micro-tuberization experiments. The data is ok if the P value is less than one and the F value is less than the F max value of 142.

3.2.1.3 % of Tubers Which Fall in each Weight Category

There do not appear to be any distinct trends for the % of tubers in each weight category. These weight categories are 0-50 mg, 50-100 mg, 100-500 mg and 500+ mg. All three estrogens and all treatments seem to have a similar percentage of tubers in each weight category throughout the entire nine experiments. There are also no outstanding differences in results of tuber weight range percentages between tubers grown with estrogen for the entire twelve weeks and those with estrogen added at the eight week stage (after four weeks growth).

A couple of points are, that in the 10 mg/L of estrogen treatments, the tubers never had the highest % in the 0-50 mg range (i.e. another treatment always had a higher % for the 0-50 mg range). Also all treatments in most of the nine experiments have the majority of their tubers in the 0-50 mg and 100-500 mg range (Figs 39-47).

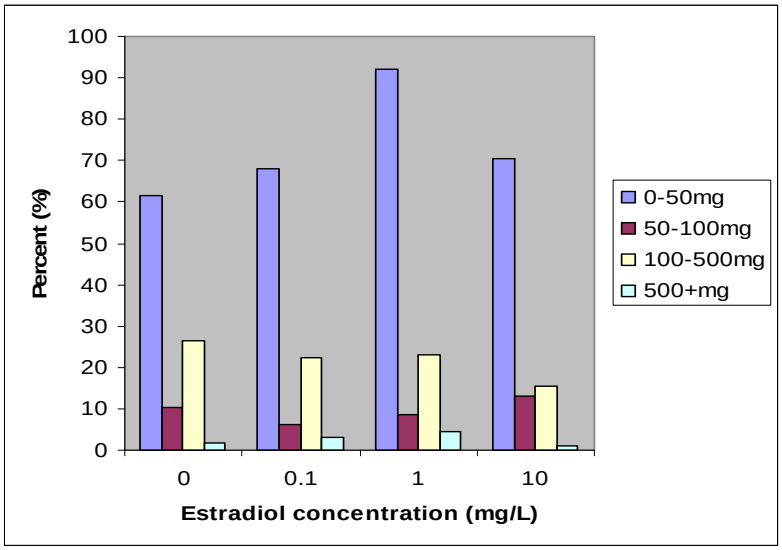


Figure 39. The % of tubers for each treatment that fall in each weight category for the 1st β-estradiol experiment.

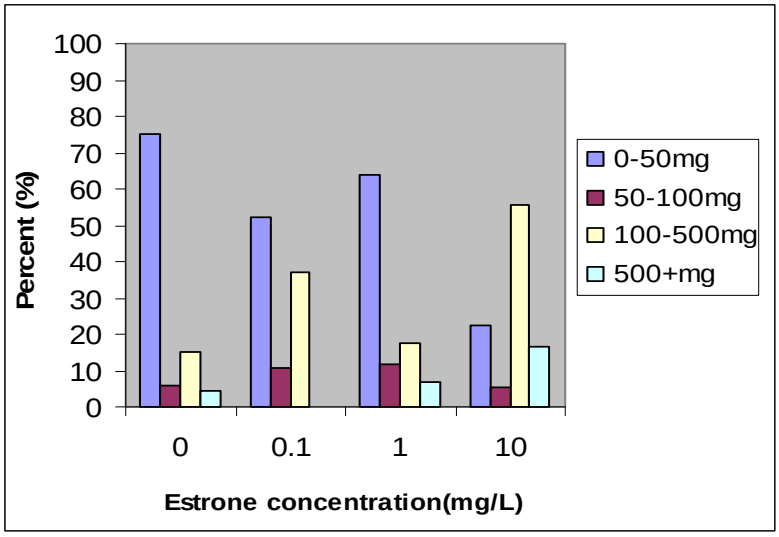


Figure 40. The % of tubers for each treatment that fall in each weight category for the 1st estrone-experiment.

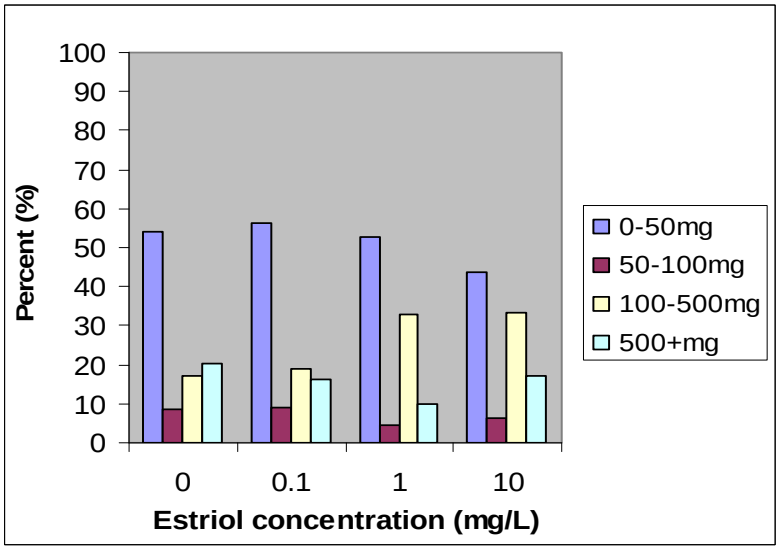


Figure 41. The % of tubers for each treatment that fall in each weight category for the 1st estriol experiment.

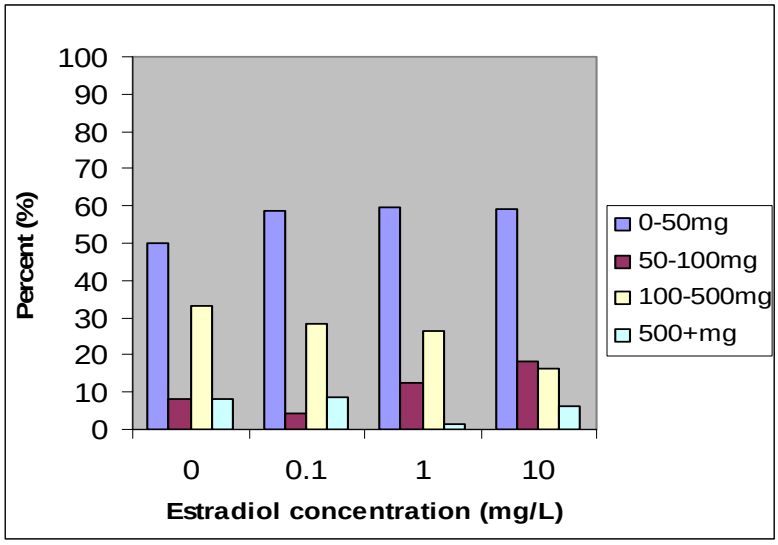


Figure 42. The % of tubers for each treatment that fall in each weight category for the 2nd β-estradiol experiment.

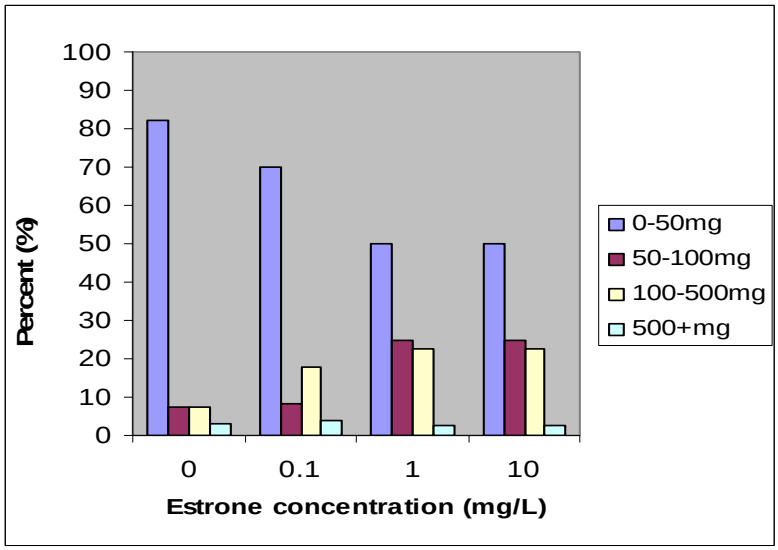


Figure 43. The % of tubers for each treatment that fall in each weight category for the 2nd estrone experiment.

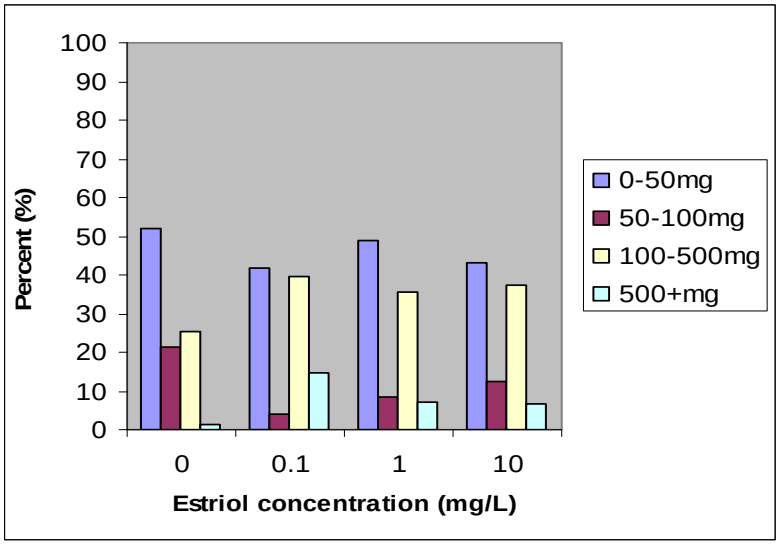


Figure 44. The % of tubers for each treatment that fall in each weight category for the 2nd estriol experiment.

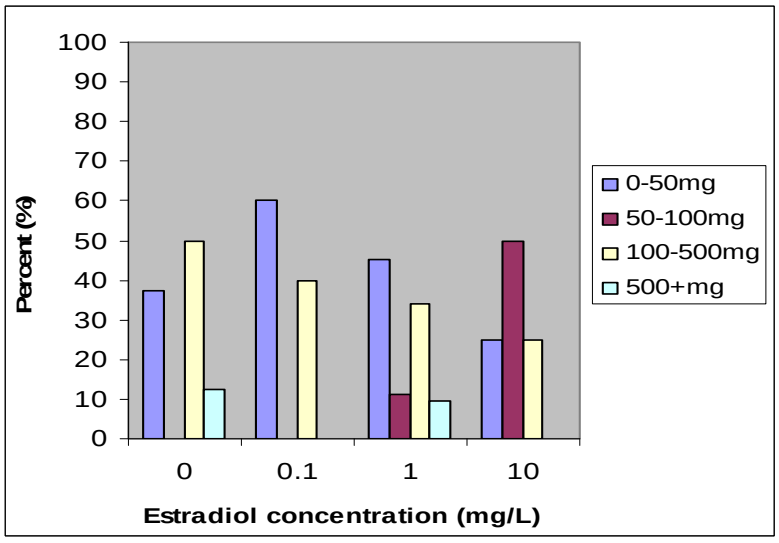


Figure 45. The % of tubers for each treatment that fall in each weight category for the 3rd β-estradiol experiment.

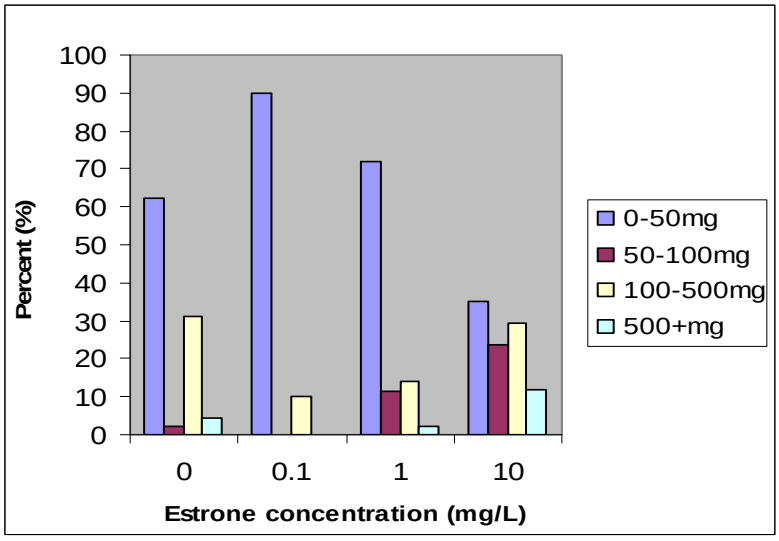


Figure 46. The % of tubers for each treatment that fall in each weight category for the 3rd estrone experiment.

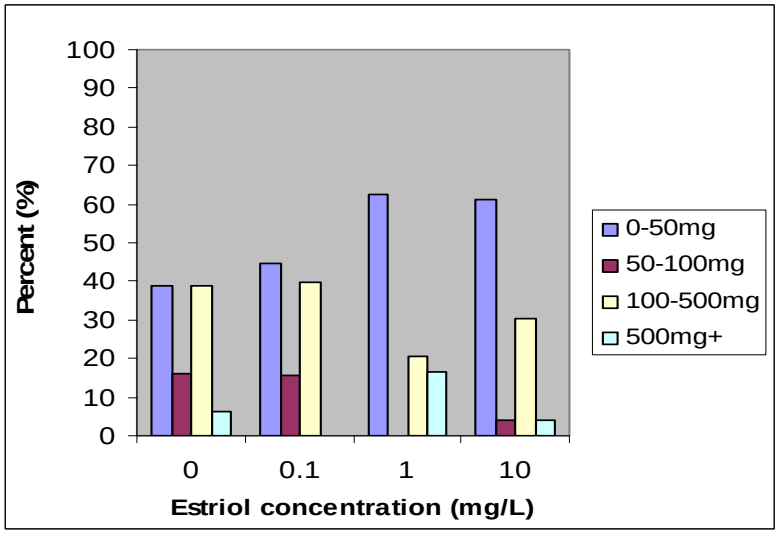


Figure 47. The % of tubers for each treatment that fall in each weight category for the 3rd estriol experiment.

3.2.2 Qualitative Results for the Microtubers

The microtubers grew on both the roots of the potato plants and the shoots. Overall, more tubers grew on the shoots of the potato plants than on the roots.

There were no real differences in appearance between tubers grown with no estrogen or those grown with 0.1, 1 and 10 mg/L of estrogen. Also there were no outstanding differences in appearance between tubers grown with β-estradiol, estrone or estriol. See plates 16-22 for examples of the tubers' appearance.

Plate 16. Example of a microtuber.

Plate 17. Example of a microtuber.

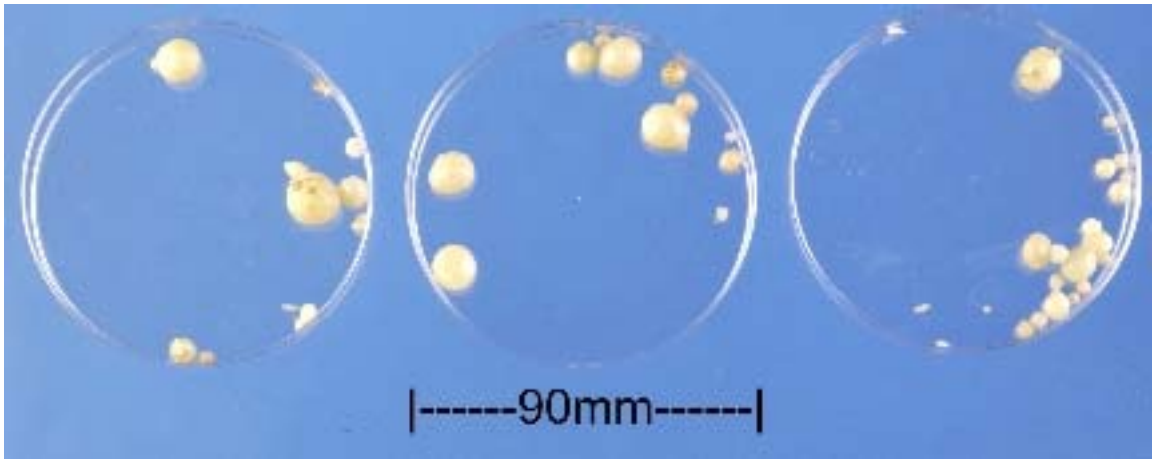


Plate 18. Example of shape and size range of microtubers from the control for the 2nd estriol experiment.

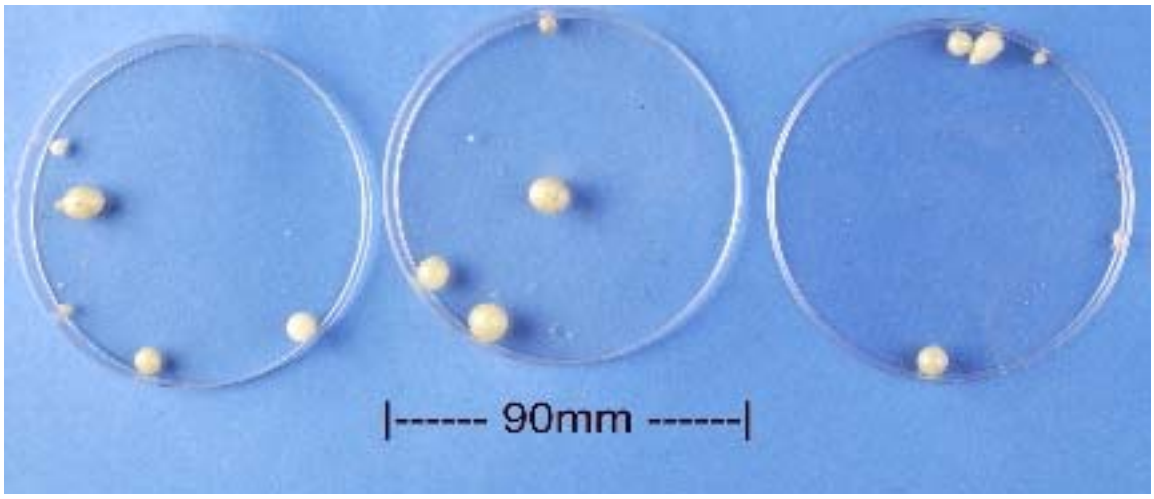


Plate 19. Example of shape and size range of microtubers from the 0.1 mg/L treatment for the 3rd β-estradiol experiment.

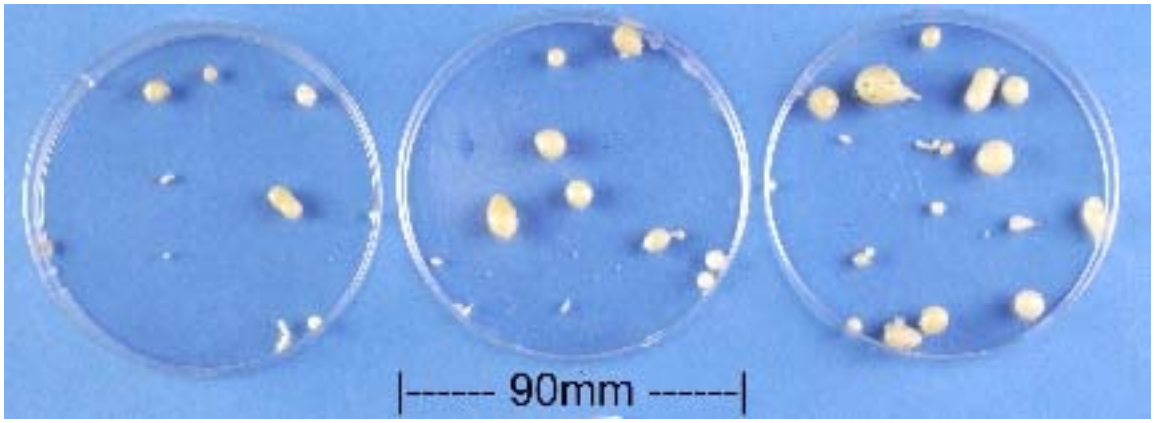


Plate 20. Example of shape and size range of microtubers from the 1 mg/L treatment for the 2nd β-estradiol experiment.

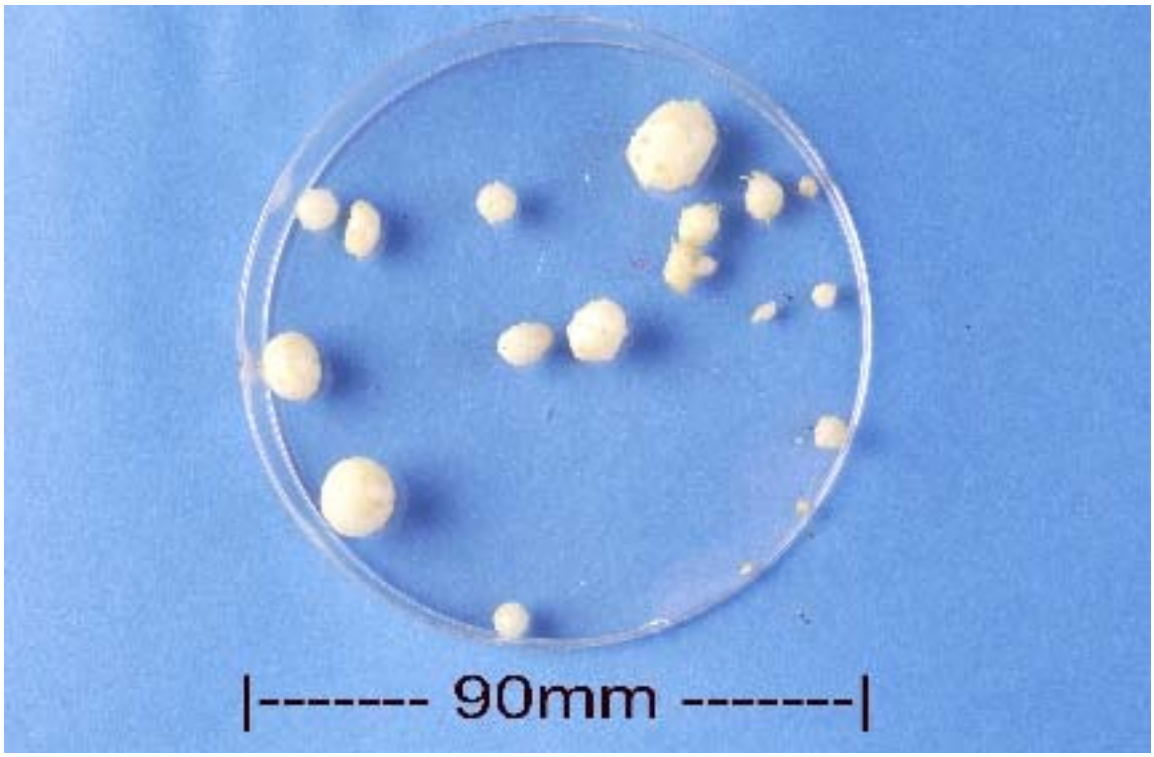


Plate 21. Example of shape and size range of microtubers from the 10 mg/L treatment for the 3rd estrone experiment.

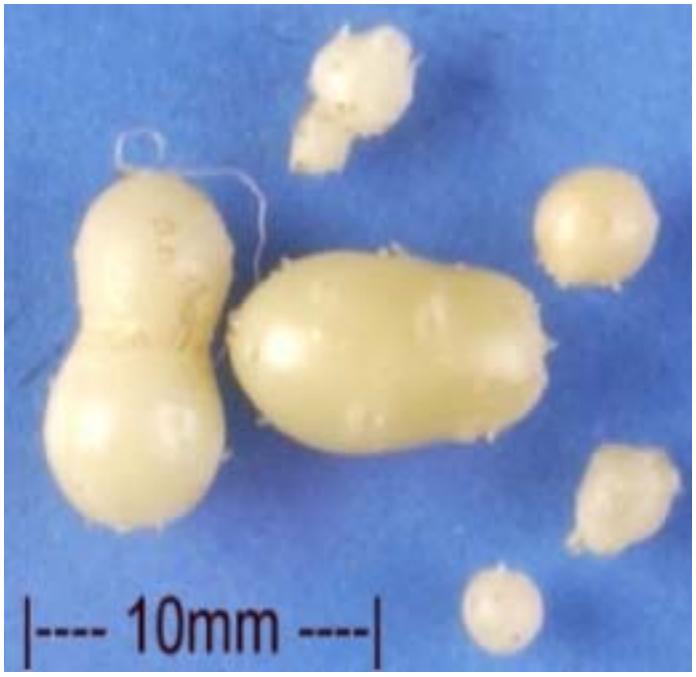


Plate 22. A close shot of some microtubers. Note the eyes (the very small indentations), on the tubers and the various shapes and sizes.

4. Discussion

4.1 Concentrations of Estrogen

As was outlined in the introduction, the aim of this research was to discover what effects environmentally relevant concentrations of mammalian estrogen would have on the growth and tuberization of potato plants grown in liquid tissue culture medium. Estrogen and estrogen mimicking compounds (xeno-estrogens) are becoming major pollutants in the environment and can be isolated at very low to very high concentrations in the environment and can be excreted at very high concentrations into the environment. The highest concentration of estrogen used in this research (10 mg/L) is a very high concentration but it was chosen as it is an environmentally relevant concentration. Land and water ways are likely to be mixed with raw human sewage (for example: pregnant women can excrete ~ 42 mg/L of β-estradiol and estrone combined in 24 hours) and animal waste (pregnant horses can excrete in excess of 200 mg in 24 hrs). The soil and plants are being exposed to herbicides and pesticides, many of which are estrogen-like compounds. For example DDT (dichlorodiphenyltrichloroethane) is an estrogen-like compound requiring years to be degraded and whose metabolite DDE (dichlorodiphenyldichloroethylene) is also a xeno-estrogen and can be found at very high concentrations i.e. 1-5 mg/ kg of soil. It is also important to use concentrations of estrogen that have been trialed in previous research (i.e. 100-1000 µg/L or 0.1-1 mg/L, for example see Shore et. al, 1992) for comparative purposes.

4.2 A Synopsis of the Significant Findings Obtained in the Present Study

In reference to the results of the dry weight of potato plant shoots, it was found that β-estradiol, estrone and estriol only had a limited effect on the vegetative growth of the potato plants even at the highest concentration of estrogen used. The common pattern that emerged from the results for the dry weight of shoots was the 0.1 mg/L and 1 mg/L concentrations of estrogen produced heavier shoots than the control and the 10 mg/L treated plants. However, all of this data does not show a statistical trend because the standard errors for each treatment overlap. The qualitative results for the effects of the three estrogens on the vegetative (shoot) growth showed a very distinct trend. The effects of estrogen on the morphology of the potato plants were not evident in the 0.1 mg/L of estrogen treatment, but in the 1 and 10 mg/L of estrogen treatments, the plants showed signs of leaf and stem deformity. Hence, higher concentrations of β-estradiol, estrone and estriol only had a very limited effect on the dry weight of the potato shoots but negatively affected the morphology of the shoots of the potato plants.

The quantitative results for the roots were basically similar to those for the shoots. The obvious effects of mammalian estrogen on the roots of potato plants were the changes to the quality of the roots (not their weight). The quality of the potato plant roots was severely affected by β-estradiol, estrone and estriol. Changes in the roots were noticeable even at the lowest concentration of estrogen. The roots of the potato plants treated with estrogen were shorter, thicker (this may contribute to a heavier weight) and less abundant. Some of the potato plants treated with 10 mg/L of estrogen appeared to have

almost no roots. The dry weights of the roots from the estrogen treated plants were not significantly lower than the dry weights of those of the control and they were sometimes higher than those of the control plant roots. This is most likely due to the fact that even though the roots were shorter and less abundant they were much thicker than those of the control. With this compensatory mechanism, in the estrogen treatments the dry weight of the roots was not different from that of the roots in the control. Hence all three mammalian estrogens experimented with affected root growth at even the lowest concentration of estrogen used.

The estrogens at the 10 mg/L concentration induced a small amount of callus growth. Callus is formed by abnormal cell growth when a plant is damaged (Dixon, 1985), so the estrogen must affect the potato plants by initiating abnormal cell growth.

Even though β-estradiol is the most potent mammalian estrogen, estrone and estriol seemed to influence the quality of the potato shoots and roots equally as severe as the β-estradiol.

The results from the acid phosphatase assays showed more clearly how estrogen is affecting the growth of the potato plants. Overall estrogen improved the acid phosphatase production in the plants treated with 0.1 mg/L and 1 mg/L of estrogen over the acid phosphatase production of the control plants. (Apart from estrone, where the 0.1 mg/L treated plants produced slightly less acid phosphatase than the control). There is a substantial decline in acid phosphatase production from the 1 mg/L treated plants to the

10 mg/L treated plants, so at the highest concentration, estrogen limits the potato plants ability to produce acid phosphatase.

The overall amount of soluble protein released into the tissue culture medium by the potato plants, followed a very similar pattern to the release of the acid phosphatase. Except the majority of the time the control plants produced more soluble protein than the plants treated with 10 mg/L of estrogen. The general trend shown was that plants treated with 10 mg/L of estrogen usually produced the least protein and those treated with 0.1 mg/L and 1 mg/L of estrogen the most protein. This was also shown in the results of the protein determination assays that showed a statistical trend in the Tukey HSD All-Pairwise Comparisons test. This means that at lower concentrations of estrogen the mammalian estrogen affects the growth of potato plants by stimulating the production and release of protein (so the metabolic function of the plants have improved) but at high concentrations of estrogen (i.e. similar to the amount of DDE that is present in soil) the metabolic functions of the plants are partially affected. The results for the protein determination assays did not always follow a regular pattern. This may be due to the possibility that small amounts of plant material (mainly minute amounts of leaf matter and loose cells from the callus formed during culture) may have been present in the tissue culture medium used for the protein assays and this could have affected the absorbance readings. To avoid any chance of this being a problem the filtration of the media would solve the issue.

An interesting fact in the tuberization experiments was the plants with no estrogen added until after four weeks of growth time all grew less tubers on average than the plants grown with estrogen for the entire twelve week period. This includes the control plants which should not have showed any major differences throughout the nine tuberization experiments. The reason for this may be to do with older stock solutions for the media, the age of the stock plants from which the cultures were taken from or the possible lighting differences. There may be lighting differences as there were more plants in the growth room at the time these plants were being grown in their four week stage. More plants in the growth room may have caused shadows to fall on some of the cultures (causing weaker plants to grow) thus resulting in a lower number of tubers being produced.

In the majority of the nine tuberization experiments, the control plants produced the highest average number of tubers and the 1 mg/L and 10 mg/L of estrogen treated plants produced the least amount of tubers. A general trend that was set was the higher the concentration of estrogen usually the less amount of tubers were produced by the potato plants. However in almost all of the nine experiments the standard errors overlapped. So the mammalian estrogens β-estradiol, estrone and estriol had a mild effect on the tuberization of potato plants by slightly reducing the number of tubers produced the higher the concentration of estrogen. A possible reason why the reduction in the number of tubers formed may have been slight (other than perhaps estrogen does not have great influence on tuberization), is that the potato plants were constantly exposed to a good amount of nutrients and carbohydrates in the tissue culture medium which are critical for

tuberization. The concentration of estrogen also seemed to affect the average dry weight per jar of tubers. The dry weight of the tubers followed a very similar pattern to the number of tubers so this may be the cause of the differences in dry weight of tubers among the treatments. As can be seen in the percentage graphs, all treatments had a similar percent (or amount of variation) of tubers in each weight category. So if there are fewer tubers then it seems logical to assume that the average dry weight of the tubers per jar would be less.

As was observed in the growth experiments, the roots at the higher concentrations of estrogen were severely affected in the tuberization experiments. How then were the tubers able to form in the plants with fewer roots? As was explained in the introduction, tubers form out of underground stems. It was observed that the micro-tubers grown in the nine tuberization experiments were also borne on the stems, many of which were not submerged in the liquid medium. The plants were in the dark during the tuberization period, so this would have simulated being in the dark under the soil in normally grown potato plants. Hence even if estrogen attacked the roots, the stems were still able to form tubers. β-estradiol, estrone and estriol did not appear to alter the morphology of the micro-tubers.

4.3 How These Results Compare to Other Research

Even though in this study the effects of estrogen on the vegetative growth of the potato plants was limited, other studies have also found that mammalian estrogen reduced shoot

growth. For example, β-estradiol and estrone at concentrations between 0.05-0.5 mg/L, which are not dissimilar to the middle two concentrations used in this experiment (0.1-1 mg/L), reduced the shoot growth of *Medicago sativa* L grown in sand with liquid nutrients (Shore et. al, 1992). In this experiment the two estrogens were also combined together to give concentrations of estrogen between 100 µg/L (0.1 mg/L) and 1000 µg/L (1 mg/L). Results from that experiment also showed reduction in shoot growth of alfalfa (Shore et. al, 1992). It is not clear how long this experiment was run for but it was at least two weeks. Morphological changes in the vegetative growth of the potato plants were noticeable within two weeks. The previous mentioned research also showed that estrogen between 50-1000 µg/L (0.05-1 mg/L) reduced root growth and those results are very similar to the qualitative results of this research where reduced root growth in the potato plants was very visible even at the lowest concentration of estrogen. In two experiments using lower concentrations of β-estradiol and estrone it was found that these estrogens reduced growth in sunflower seedlings and tomato seedlings (grown in nutrient solution)(Janeczko and Skoczowski, 2005). Even though the estrogen content in the treatments for the sunflower and tomato seedlings was lower than the concentrations used in this research, it is obvious that estrogen attacks root growth in a wide range of plants including in potato plants as is shown in the present study. Moreover, while confirming previous research showing that estrogens can reduce plant growth, this research also showed that at 10 mg/L of estrogen, acid phosphatase, protein determination and tuber production (growth indicators) were reduced.

Callus growth occurred in the potato plants treated with 10 mg/L of all three estrogens. In an experiment using 3-12 mg/L of 17-β-estradiol, it was found that the β-estradiol promoted callus growth in *Daucus carota* L. (Janeczko and Skoczowski, 2005). It has also been found that estrogen will induce callus growth in *Polygonatum verticillatum* L. (Janeczko and Skoczowski, 2005).

4.4 Further Study Possibilities

Previous research on the effects of estrogen shows that estrogen will affect flowering, pollen tube growth and germination (Janeczko and Skoczowski, 2005; Ylstra et. al, 1995). So it would be interesting to find out what effects β-estradiol, estrone and estriol would have on the flowering, sexual aspects of the flower and germination of potato plants. It is also possible that estrogen affects how much water the potato plants hold, so testing the percentage of water loss from fresh to dry weight may indicate how well plants assimilate water when being exposed to estrogen. With the morphology of stems, leaves and roots being altered when they have been exposed to estrogen it is highly likely that the individual plant cells have altered, so microscopic investigation of potato plant cells and tuber cells may show that estrogen affects the entire structure of the potato plant and may affect the internal tissue/cellular organization of tubers. The data from the growth experiments were obtained after four weeks in the present study; it would be interesting to see what effect estrogen may have in shorter time periods i.e. five days or longer time periods i.e. twelve weeks. The concentrations of estrogen used for this research were quite high even though they were very relevant concentrations.

Investigation of the effects of estrogen of the growth and tuberization of potato plants using much lower concentrations of estrogen is important, also using a wider range of estrogen, synthetic estrogens and xeno-estrogens would give a broader example of how estrogens can affect the growing aspects and tuberization of potato plants. Research on the effects of very low concentrations of estrogen on the growth of potato plants is important because other researchers have found that very low concentrations (i.e. 0.005 μg/L) increases plant growth (Shore et. al, 1992; Janeczko and Skoczowski, 2005). If estrogen at low concentrations improved growth of the potato plants this may be important to the agricultural industry by promoting better plant growth.

The mechanism(s) by which estrogen can influence plant growth and development is unknown at present. Would the estrogen be taken up by plant roots? How much estrogen and possibly their metabolites might be transported to the shoots? Is it necessary for the estrogen to be taken up to the shoot before it can affect stem morphology? These are somewhat unanswered questions. Given that plant growth and development is naturally under the regulation of various plant hormones (Plant Physiology 2nd Ed, 1998), it is tempting to speculate that exposure to estrogen in the environment could cross-walk with endogenous plant hormones. Future studies should be designed to gain new insights about this.

4.5 Conclusion

Even though concentrations of estrogen used and time periods of previous research may be at variance from this research, the overwhelming consensus is that mammalian estrogens (β-estradiol, estrone and estriol) do affect plant growth. This research has used environmentally relevant concentrations of estrogen and we have found that estrogen can have an effect the morphology of plant, can limit root growth and limit the metabolic functions of potato plants. Estrogen appeared to have a limited effect on the tuberization of the potato plants grown in tissue culture medium but it still reduced the overall tuber production of the plants. Since estrogen is becoming a major pollutant, more research into the effects of mammalian estrogen on plants is necessary.

Reference

- Aislabie JM, Richards NK, Boul HL (1997). Microbial degradation of DDT and its residues- a review. NZ Journal of Agriculture Research 40: 269-282
- Angosto T, González F, Matilla A. (1988). Partial purification and some biochemical properties of acid phosphatases in germinating chickpea (*Cicer arietinum*) seeds. Physiol. Planta. 74: 715-719
- Aoyama H, Cavagis ADM, Taga EM, Ferreira CV. (2001). Endogenous lectin as a possible regulator of the hydrolysis of physiological substrates by soybean seed acid phosphatase. Phytochemistry 58: 221-225
- Atkinson S, Alkinson MJ, Tarrant AM. (2003). Estrogens from sewage in coastal marine environments. Environmental Health Perspectives vol. 111 no. 4.
- Baldwin JC, Karthikeyan AS, Raghothama KG. (2001). LEPS2, a phosphorus starvation-induced novel acid phosphatase from tomato. Plant Physiol. 125: 728-737
- Bieleski RL. (1973). Phosphate pools, phosphate transport and phosphate availability. Annual Review Plant Physiol. 24: 225-252
- Bourgaize D, Jewell TR, Buiser RG. (2000). Biotechnology. Demystifying the Concepts pp. 356-357
- Bradford MM. (1976). A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochem. 72: 248-254
- Dakora FD, Phillips DA. (2002). Root exudates as mediators of mineral acquisition in low-nutrient environments. Plant and Soil 245: 35-47

- Dixon RA. (1985). Plant cell culture: a practical approach. Oxford, IRL.
- Diz MSS, Carvalho AO, Gomes VA. (2003). Purification and molecular mass determination of a lipid transfer protein exuded from *Vigna unguiculata* seeds. Brazilian Journal of Plant Physiol. 15 (3)
- Duff SMG, Lefebvre DD, Plaxton WC. (1989). Purification and characterization of phosphoenol-pyruvate phosphatase from *Brassica nigra* suspension cells. Plant Physiol. 90: 734-741
- Duff SMG, Sarath G, Plaxton WC. (1994).The role of acid phosphatases in plant phosphorus metabolism. Physiol. Planta. 90: 791-800
- Ehsanpour AA and Amini F. (2003). Effects of salt and drought stress on acid phosphatase activity in alfalfa (*Medicago sativa* L.) explants under in vitro culture. African Journal of Biotechnology. 2(5) : 133-135
- Fenske M, Maack G, Schäfers C, Segner H. (2005). An environmentally relevant concentration of estrogen induces arrest of male gonad development in Zebra fish, *Danio rerio*. Environmental Toxicology and Chemistry vol.24 no.5 : 1088-1098
- Funk and Wagnall's New Encyclopaedia (1990) vol 9: 237 and 367. Funk and Wagnall's Corporation
- Gleba YY, Hoffmann F. (1980). "Arabidobrassica". A novel plant obtained by protoplast fusion. Planta. 149(2): 112-117
- Guan M, Roddick JG. (1988). Comparison of the effects of epibrassinolide and steroidal estrogens on adventitious root growth and early shoot development in mung bean cuttings. Physiol. Plant 73: 426-431

- Guidelines on the Use of Urine and Faeces in Crop Production. EcoSanRes fact sheet 6 (2005). Stockholm Environment Institute. Sourced from: www.ecosanres.org
- http://gamma.mbb.ki.se/~tanja/pap.hyml
- http://www.britannica.com/eb/article-9061051?query=potato&ct
- http://www.nrcan.gc.ca/cfs/biotech/treepr_e.html
- Janeczko A, Filek W, Biesaga-Kościelniak J, Marcińska I, Janeczko Z. (2003). The influence of animal sex hormones on the induction and flowering in *Arabidopsis thaliana:* a comparison with the effect of 24-epibrassinolide. Plant Cell, Tissue and Organ Culture 72: 147-151
- Janeczko A, Skoczowski A. (2005). Mammalian sex hormones in plants. Folia Histochemica ET Cytobiologica. 43(2): 71-79
- Ling N. (2002). A study on rhizosecretion during micro-propagation of potato (*Solanum tuberosum* L. cv. Iwa). A thesis submitted in partial fulfilment of the requirements for the degree of Masters of Science at the University of Canterbury 2002.
- Matsuda T, Matsui S, Takigami H, Adachi J, Ou S. (2001). Separation of urine from municipal sewage for prevention of water pollution. Internet Dialogue on Ecological Sanitation (15^{th} Nov-20^{th} Dec, 2001). Research Centre for Environmental Quality Control, Kyoto University
- Matthews CK, van Holde KE. (1990). Biochemistry. The Benjamin/Cummings Publishing Company Inc. 1990 ©

- Murashige T, Skoog F. (1962). A revised medium for rapid growth and bio assays with tobacco tissue cultures. Plant Physiol. 15: 473-497
- Pijnacker LP, Ferwerda MA. (1990). Effects of sucrose on polyploidization in early callus cultures of *Solanum tuberosum*. Plant Cell, Tissue and Organ Culture. 21(2): 153-157
- Potato Association of American Handbook. (2005). Sourced from: http://cropandsoil.oregonstate.edu/classes/CSS322/Introwp.htm
- Randall D, Burggren W, French K. (2001). Eckert Animal Physiology-Mechanisms and Adaptations 5th Ed. W.H. Freeman and Company New York pp 301-360
- Raven PH, Evert RF, Eichhorn SE. (1999). Biology of Plants 6th Ed. W.H. Freeman and Company Worth Publishers pp. 693-695
- Seeni S, Latha PG. (1991). Foliar regeneration of the endangered Red Vanda, *Renanthera imschootiana* Rolfe (Orchidaceae). Plant Cell, Tissue and Organ Culture. 29(3): 167-172
- Shaw I. (2001). Food safety-A 21st century issue. NZ Science Review 58(2): 38-46
- Shore LS, Gurevitz M, Shemesh M. (1993). Estrogen as an environmental pollutant. Bulletin of Environmental Contamination and Toxicology. 51: 361-366
- Shore LS, Kapulnik Y, Ben-Dor B, Fridman Y, Wininger S, Shemesh M. (1992). Effects of estrone and 17-β-estradiol on vegetative growth of *Medicago sativa*. Physiol. Planta. 84: 217-222

- Shore LS and Shemesh M. (2003). Topic 2.2 naturally produced steroid hormones and their release into the environment. Pure and Applied Chemistry 75: 1859-1871
- Staswick PE, Chuck P, Jing-Feng H, Yoon P. (1994). Purification of the major soybean leaf acid phosphatase that is increased by seed-pod removal. Plant Physiol. 104: 49-57
- Taiz L, Zeiger E. (1998). Plant Physiology 2nd Ed. Sinauer Associates Inc., Publishers. Massachusetts. pp. 472 and 622
- The Merck Index: An Encyclopaedia of Chemicals, Drugs and Biologicals 11th Ed. (Centennial Ed.) (1989). Editor: S Budavari. Published by Merck and Co. Inc. Rahway New Jersey USA. pp. 584-585
- Thomson BM. (2005). Human health implications of exposure to xeno-estrogens from food. A thesis submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Chemistry at the University of Canterbury 2005.
- Thomson BM, Cressey PJ, Shaw IC. (2003). Dietary exposure to xeno-estrogens in New Zealand. Journal of Environ. Monit. 5: 229-235
- Tomscha JL, Trull MC, Deikman J, Lynch JP, Guiltinan MJ. (2004). Phosphatase under-producer mutants have altered phosphorus relations. Plant Physiol. 135: 334-345
- Turner WL, Plaxton WC. (2001). Purification and characterization of banana fruit acid phosphatase. Planta 214: 243-249

- Vreugdenhil D, Van Dijk W. (1989). Effects of ethylene on the tuberization of potato (*Solanum tuberosum*) cuttings. Plant Growth Regulation 8(1): 31-39
- Yan X, Liao H, Trull MC, Beebe SE, Lynch JP. (2001). Induction of a major leaf acid phosphatase does not confer adaption to low phosphorus availability in common bean. Plant Physiol. 125: 1901-1911
- Ylstra B, Touraev A, Brinkmann AO, Herberle-Bors E, van Tunen AJ. (1995). Steroid hormones stimulate germination and tuber growth of in vitro matured tobacco pollen. Plant Physiol. 107: 639-643
- Yoon KS. (2000). Investigation of a two-step process for potato (*Solanum tuberosum* L.) microtuber production. A thesis submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy at the University of Canterbury in 2000.
- Yu WC, Joyce PJ, Cameron DC, McCowan BH. (2000). Sucrose utilization during microtuber growth in bioreactors. Plant Cell Reports. 19(4): 407-413

Acknowledgments

I would like to thank my Dad, Mum and Alexander (my brother), Aunty Middy (who kindly offered to type for me if I ran out of time but luckily I didn't as I doubt she could have read my appalling hand writing), Aunty Kathy and many other family members and friends for their support during the time it has taken me to complete my M.Sc. thesis. Even though at times they were clearly bored and tired of listening to my constant ramblings (I could tell due to that glazed look one gets in the eyes when going through awful experiences such as watching other people's holidays on home video), they still supported and encouraged me.

A HUGE thankyou must go to my supervisor Dr. David Leung. Thank you for all your help and always being around to help us in the Biotech lab, even when you were busy dealing with other matters and conducting your own "Top secret" research. Also thanks to my co-supervisor Dr. Paul Broady. A big thanks to all my lab mates, thanks for all your friendships and help and I hope we all keep in touch. A special thanks to Lily and Ying who gave me plenty of assistance when I hadn't a clue to what I was supposed to be doing. Thankyou to Nicole, Craig, Matt, Graham and all the other staff members of the Biological Sciences department whose constant work made life much easier for me during my time of research. Thank you to Dr. Anna Janeczko and Dr. Laurence Shore who provided me with information on previous research. Also thanks to those who said I should go and get a real occupation (instead of being a student) or that I would never make it through high school. You made me more determined to pursue a higher education. Thankyou to God for allowing me the opportunity to complete an education I thought would only be a dream. SO THANKS ALL……

APPENDIX 1. Murashige and Skoog (MS) Tissue Culture Media

4 stock solutions are required and amounts shown are for 500 ml of solution.

1). Major Salts (500ml)

NH_4NO_3	8.25 g
KNO_3	9.5 g
$CaCl_2.2H_2O$	2.2 g
$MgSO_4.7H_2O$	1.85 g
KH_2PO_4	0.85 g

Bring to volume (500 ml) with dH_2O (distilled water).

Store at 4˚C

2). Minor Salts (500 ml)

KI	0.0415 g
H_3BO_3	0.310 g
$MnSO_4.4\ H_2O$	1.115 g
$ZnSO_4.7\ H_2O$	0.43 g
$CuSO_4.5\ H_2O$	0.00125 g
$CoCl_2.\ 6\ H_2O$	0.00125 g
$Na_2MoO_4.2\ H_2O$	0.0125 g

Bring to volume (500 ml) with dH_2O.Store at 4˚C

3). Organic Supplement (500 ml)

Myo-inositol	5 g
Nicotinic acid	0.025 g
Pyridoxine-HCl	0.025 g
Thiamine-HCl	0.005 g
Glycine	0.1 g

Bring to volume (500 ml) with dH_2O.

Store at 4˚C

4). Iron Stock (500 ml)

Solution A: $FeSO_4.7H_2O$	1.39 g in 200 ml of dH_2O
Solution B: $Na_2EDTA.2H_2O$	1.865 g in 200 ml of dH_2O

Mix solution A and solution B.

Adjust volume to 500 ml with dH_2O.

Store in a dark bottle at 4˚C

Amount of Stock Solutions Required for Making the Media

	200 ml	**500 ml**	**1 Litre**
dH2O	150 ml	375 ml	750 ml
Major Salts	20 ml	50 ml	100 ml
Minor Salts	2 ml	5 ml	10 ml
Organic Supplement	2 ml	5 ml	10 ml
Iron Stock	2 ml	5 ml	10 ml

1). Mix the stock solutions and dH_2O together.

2). Add the required amount of sucrose and stir until dissolved (i.e. if 3 % (w/v) is required that would be 15 g for 500 ml of media and 8 % (w/v) would be 40 g for 500 ml of media).

3). Set pH of media to 5.6-5.8

4). Add agar (1.6 g for 200 ml of media, 4 g for 500 ml of media and 8 g for 1 Litre of media) and heat to dissolve the agar. For liquid media leave the agar out and no heating is required.

5). Dispense the media.

6). Autoclave the media.

APPENDIX 2. Estrogen Stock Solutions

The stock solutions are made for 80 µl of stock solution per 40 ml of tissue culture media.

1). 10 mg/L of estrogen: Dissolve 5 mg of the required estrogen in 1 ml of 100 % (v/v) ethanol. (So 2 ml of this stock solution would be required per Litre for a 10 mg/L concentration and since the volume of media is 40 ml, 0.08 ml which is 80 µl is needed per 40 ml for 10 mg/L of estrogen).

2). 1 mg/L of estrogen: Take 0.1 ml of stock solution one (the 10 mg/L concentration) and bring to 1 ml volume using 100 % (v/v) ethanol. (So 2 ml of this stock solution would be required per Litre for a 1 mg/L concentration and since the volume of media is 40 ml, 0.08 ml which is 80 µl is needed per 40 ml for 1 mg/L of estrogen).

3). 0.1 mg/L of estrogen: Take 0.1 ml of stock solution two (the 1 mg/L concentration) and bring to 1 ml volume using 100 % (v/v) ethanol. (So 2 ml of this stock solution would be required per Litre for a 0.1 mg/L concentration and since the volume of media is 40 ml, 0.08 ml which is 80 µl is needed per 40 ml for 0.1 mg/L of estrogen).

4). 0 mg/L of estrogen: just use 100 % (v/v) ethanol.

APPENDIX 3. Citrate Buffer pH 5.0

Two stock solutions are required.

Stock solution A: 0.1 M solution of citric acid = 21.01 g of citric acid dissolved in 1000 ml of dH_2O (distilled water).

Stock solution B: 0.1 M solution of sodium citrate ($C_6H_5O_7Na_3.2H_2O$) = 29.41 g of sodium citrate dissolved in 1000 ml of dH_2O.

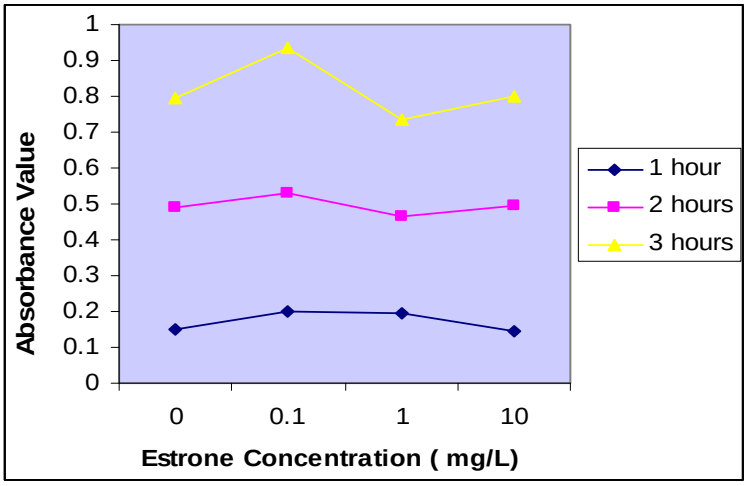
To make 100 ml of pH 5.0 citrate buffer mix 20.5 ml of stock solution A with 29.5 ml of stock solution B, then dilute to a total of 100 ml using dH_2O.

APPENDIX 4. The Trial Run Acid Phosphatase Assays

These are only the absorbance readings from the acid phosphatase assays that were carried out to establish a suitable time period for incubation. The absorbance readings are all that were required to establish this time period. Control assays were performed but were not required to decide the incubation period so they have not been included in this Appendix. The media used for these trial assays came from the estrone trial run growth experiment.

	Absorbance Readings at:		
Estrone concentration	**1 Hour**	**2 Hours**	**3 Hours**
0 mg/L	0.150	0.489	0.796
0.1 mg/L	0.198	0.528	0.934
1 mg/L	0.194	0.466	0.733
10 mg/L	0.146	0.495	0.798

Appendix 4 Table 1. The average absorbance readings from the trial acid phosphatase assays used to determine whether 1, 2 or 3 hours incubation at 37˚C was the most suitable incubation time.



Appendix 4 Figure 1. The absorbance values for the trial acid phosphatase assays from three different incubation time periods.

APPENDIX 5. Bradford Reagent

The following instructions are for making 1000 ml of Bradford Reagent.

Dissolve 100 mg of Coomassie Brilliant Blue G-250 (sigma) in 50 ml of 95 % ethanol. Add to this solution 100 ml of 85 % (w/v) phosphoric acid. Finally dilute the solution to 1000 ml.

The final concentrations of the various ingredients in the Bradford Reagent will be: 0.01 % (w/v) Coomassie Brilliant Blue G-250, 4.7 % (w/v) ethanol and 8.5 % (w/v) phosphoric acid. (Bradford, 1976).

APPENDIX 6. Tuber's Trial

Here are the results to the experiment used to establish the correct technique for initiating tuber formation in tissue cultured potato plants.

Replicate	No. of tubers
1	2
2	10
3	0

Appendix 6 Table 1. As can be seen the methods trialed to initiate tuberization in the tissue cultured potato plants were successful as tubers were produced. The plants from replicate three died hence no tuber production.